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אני, (שם, המבקש, מענו ולגבי גוף מאוגד - מקום התאגדותו)
I (Name and address of applicant, and in case of body corporate - place of incorporation)

(50%)YISSUM RESEARCH DEVELOPMENT COMPANY OF THE HEBREW UNIVERSITY OF JERUSALEM
P.O. Box 39135
Jerusalem 91390
(50%)TEL AVIV SOURASKY MEDICAL CENTER
6 WEIZMANN STREET
Tel Aviv 64239

(50%) יישום חברה לפיתוח המחקר של האוניברסיטה העברית בירושלים
ת.ד. 39135
ירושלים 91390
(50%) המרכז הרפואי תל אביב ע"ש סוראסקי
רחוב וייצמן 6
תל אביב 64239

Inventors: Hermona Soreq
Perry Chava
Grisaru Dani
Pick Marjorie
Deutsch Varda

ממציאים: שורק חרמונה
פרי חוה
גריסרו דני
מרג'ורי פיק
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בעל אמצאה מכח הדין

an invention the title of which is
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ששמה הוא
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ARP כגורם המשרה גרנולוציטופוייזה, שימושים ושיטות

(English)

(באנגלית)

ARP AS AN INDUCER OF GRANULOCYTOPOIESIS, USES AND METHODS THEREOF

hereby apply for a patent to be granted to me in respect thereof.

מבקש בזאת כי ינתן עליה פטנט

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המען למסירת מסמכים בישראל
Address for Service in Israel
LUZZATTO & LUZZATTO לוצאטו את לוצאטו
P.O. Box 5352 ת.ד. 5352
Beer-Sheva 84152 באר שבע 84152

חתימת המבקש
Signature of Applicant
Shel Zuli'y
לוצאטו את לוצאטו
ב"כ המבקשת
LUZZATTO & LUZZATTO
ATTORNEYS FOR APPLICANTS

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ARP כגורם המשרה גרנולוציטופוייזה, שימושים ושיטות

ARP AS AN INDUCER OF GRANULOCYTOPOIESIS, USES AND METHODS THEREOF

Field of the Invention

The present invention relates generally to the field of hematopoiesis and more specifically to the effect of an AChE-derived peptide on different hematopoietic sub-populations.

Background of the Invention

All publications mentioned throughout this application are fully incorporated herein by reference, including all references cited therein.

Mammalian hematopoietic stem cells develop during embryogenesis and differentiate into the different hematopoietic lineages [Tavian, M. *et al.* (2001) *Immunity* **15**:487-95]. After birth, the capacity of myeloid cells to respond to external and/or internal stimuli by the finely tuned production of pro-inflammatory and anti-inflammatory cytokines [Shanks, N., and S. L. Lightman (2001) *J. Clin. Invest.* **108**:1567-73] is gradually acquired [Hanada, T., and A. Yoshimura (2002) *Cytokine Growth Factor Rev.* **13**:413-21], in parallel with the establishment of fully mature lymphocytic immune responses [de Vries, E. *et al.* (2000) *Pediatr. Res.* **47**:528-37]. Interestingly, the responses of both myeloid and lymphoid cell lineages are subject to acetylcholine (ACh) modulation [Kawashima, K., and T. Fujii (2000) *Pharmacol. Ther.* **86**:29-48; Tracey, K. J. (2002) *Nature* **420**:853-9], which involves the α7 nicotinic ACh receptor [Wang, H. *et al.* (2003) *Nature* **421**:384-8] and are known to be impaired under psychological stress [Miller, G. E. *et al.* (2002) *Health Psychol* **21**:531-41]. However, the putative protein(s) mediating these developmental and stress-induced processes is yet unknown.

Peripherally acting acetylcholine (ACh) was recently shown to control pro-inflammatory responses [Tracey (2002) *id ibid.*]. ACh levels, in turn, are controlled by circulating cholinesterases, and particularly acetylcholinesterase (AChE). The *ACHE* gene contains a functional glucocorticoid response element (GRE) in its distal promoter and multiple putative binding sites for hematopoiesis-related transcription factors [Deutsch, V.R. *et al.* (2002) *Exp.*

Hematol. **30**:1153-1161]. Alternative splicing gives rise to the "synaptic" (AChE-S) multimers, the "erythrocytic" (AChE-E) dimers and the stress-induced "readthrough" (AChE-R) monomers [Grisaru, D. *et al.* (1999a) *Eur. J. Biochem.* **264**:672-686; Karpel, R. *et al.* (1994) *Exp. Cell Res.* **210**:268-277]. AChE-R is expressed in multiple embryonic and tumor cells, where it displays morphogenic functions, but is rarely found in healthy and unstressed adult tissues [Grisaru (1999a) *id ibid.*; Karpel (1994) *id ibid.*; Soreq, H., and S. Seidman (2001) *Nat. Rev. Neurosci.* 2:294-302; Grisaru, D. *et al.* (1999b) *Mol. Cell Biol.* **19**:788-795] or human sera [Brenner *et al.* (2003) *FASEB J.* **17(2)**:214-22]. Cortisol induces AChE-R production in cultured CD34+ blood cell progenitors [Grisaru, D. *et al.* (2001) *Molecular Medicine* 7:93-105]. ARP_{26}, a synthetic peptide designed to mimic the cleavable C-terminal sequence of AChE-R, promotes hematopoietic proliferation *in vitro* [Grisaru (2001) *id ibid.*].

Within their individual microenvironment, blood cells receive external stimuli which influence transcription and processing of many reactive molecules. Particular alternatively spliced AChE variants may be candidates to exert both enzymatic and non-catalytic functions on these cells. The expression of AChE-S in blood cells has been associated with terminal differentiation [Chan, R. Y. Y. *et al.* (1998) *J. Biol. Chem.* **273**:9727-9733] and apoptosis [Zhang, X. J. *et al.* (2002) *Cell Death Differ.* **9**:790-800]. In contrast, AChE-R and a synthetic peptide with the sequence of the unique C-terminal residues of AChE-R, were associated with stem myeloid cell proliferation [Grisaru (2001) *id ibid.*; Deutsch *et al.* (2002) *id ibid.*]. To correctly evaluate the potential contribution of AChE towards differentiation, proliferative or apoptotic events in hematopoiesis, and in inflammatory responses under stress stimuli, specific variants were identified, their levels quantified and their subcellular localization (i.e. on the cell surface and/or intracellular) determined in specific blood cell lineages. For this purpose the inventors combined the use of flow cytometry of human peripheral blood cells and plasmon resonance analysis of blood cell extracts, using selective antibodies raised against the C-terminal

peptides of AChE-S [Flores-Flores, C. *et al.* (2002) *J. Neural Transm.* **62(suppl):**165-179] and AChE-R [Sternfeld, M. *et al.* (2000) *Proc. Natl. Acad. Sci. U.S.A.* **97:**8647-52].

Post-stress leukocytosis, i.e. overproduction of white blood cells (WBC), was first described over 50 years ago [Milhorat, A. *et al.* (1942) *Arch. Neurol. Psychiatr.* **47:** 779-784]. Elevated WBC counts occur after diverse stress insults, e.g. shock, blood loss, in post-partum mothers or following space flight or bacterial infection [Delgado, I. *et al.* (1994) *Gynecol. Obstet. Invest.* **38:** 227-235; Reizenstein, P. (1979) *Br. J. Haematol.* **43:** 329-334; Stowe, R.P. *et al.* (1999) *J. Leukoc. Biol.* **65:** 179-186; Toft, P. *et al.* (1994) *Apmis* **102:** 43-48; Wanahita, A. *et al.* (2002) *Clin. Infect. Dis.* **34:** 1585-1592]. The initiation of WBC overproduction has been attributed to the elevated serum levels of cortisol [Laakko, T. and Fraker, P. (2002) *Immunology* **105:** 111-119], causing both enhanced proliferation and facilitated WBC maturation, predominantly toward the granulocytic lineage [Abramson, N. and Melton, B. (2000) *Am. Fam. Physician* **62:** 2053-2060]. However, the increased levels of cortisol, e.g. following the stressful event of delivery, recede within a few hours [Tuimala, R. *et al.* (1976) *Br. J. Obstet. Gynaecol.* **83:** 707-710], and cannot account for the prolongation of leukocytosis, especially since the lifespan of granulocytes is extremely short, with 50% of the granulocytes being replaced by the bone marrow daily [Abo, T. and Kawamura, T. (2002) *Ther. Apher.* **6:** 348-357]. The signaling pathways controlling this process therefore remain largely unknown.

Granulocytosis depends upon the production of pro-inflammatory/hematopoietic cytokines which in peripheral tissues is regulated by acetylcholine (ACh) [Borovikova, L.V. *et al.* (2000) *Nature* **405:** 458-462; Tracey, K.J. (2002) *Nature* **420:** 853-859]. Under normal conditions, ACh activates α7 ACh nicotinic receptors on macrophages to attenuate pro-inflammatory cytokine secretion at the post-transcriptional level [Wang, H. (2003) *id ibid.*]. However, in aging patients the capacity to suppress the production of pro-inflammatory cytokines decreases, leading to age-induced

increases in serum IL-6, for example [Kiecolt-Glaser, J. K. *et al.* (2003) *Proc. Natl. Acad. Sci. U.S.A.* **100**(15) 9090-5]. To determine whether post-stress ACh levels can account for the prolonged granulocytosis effect independently of cortisol, and to delineate the cascade of events that enables this process, the inventors studied circulating acetylcholinesterase (AChE) [Soreq and Seidman (2001) *id ibid.*]. Agents performing this reaction can further be used to control the production of cytokines in patients with failure of such responses. The *ACHE* gene contains a functional glucocorticoid response element (GRE) in its distal promoter [Meshorer *et al.* (2002) *Science* **295**:508-12] and multiple putative binding sites for hematopoiesis-related transcription factors [Grisaru *et al.* (2001) *id ibid.*].

Cortisol induces AChE-R production in cultured CD34+ cells [Grisaru *et al.* (2001) *id ibid.*]. Therefore, the inventors considered, as a working hypothesis, circulating AChE-R to be a modulator of sustained granulocytosis effects in hematopoietic progenitors. The demonstrated involvement of AChE-R in both hematopoietic and neuronal [Kaufer *et al.* (1998) *id ibid.*; Meshorer *et al.* (2002) *id ibid.*] stress responses supported this assumption. Under stress responses, AChE-R undergoes C-terminal cleavage in both mice [Grisaru *et al.* (2002) *id ibid.*] and humans [Cohen *et al.* (2003) *id ibid.*]. In cell cultures, ARP_{26}, a synthetic peptide having the sequence of the C-terminus of AChE-R, operates as a hematopoietic growth factor [Deutsch *et al.* (2002) *id ibid.*; Grisaru *et al.* (2001) *id ibid.*]. To find out whether AChE-R and/or ARP are associated with post-stress granulocytosis and cytokine production, the inventors initiated a study aimed at delineating the *in vivo* and *ex vivo* regulation of AChE-R production in stress-induced myelopoietic processes.

Thus, an aim of the present invention is to provide novel uses for an AChE-derived peptide, as an agent capable of inducing granulopoiesis, as demonstrated in the following Examples.

It is another aim of the present invention to provide a method for the treatment of conditions that induce a low granulocytic cell count, administering said AChE-derived peptide, and compositions thereof, to a subject in need.

Further, the present invention provides methods of evaluating lymphocytic activity, based on the expression of the different AChE forms on lymphocytes.

Other purposes and advantages of the invention will appear as the description proceeds.

Summary of the Invention

The inventors have demonstrated that overproduction and C-terminal cleavage of the stress-induced AChE-R isoform induced long-lasting granulocytosis, independently of cortisol levels.

In this view, in a first aspect, the present invention provides the use of an AChE-derived peptide as an agent for the induction of the production of granulocytes, wherein said peptide is denoted by SEQ. ID. NO.1. The peptide used by the invention comprises the following amino acid sequence:

N' – GMQGPAGSGWEEGSGSPPGVTPLFSP – C'

Said peptide may also be an agent for the induction of repopulation and/or rematuration of granulocytic cell populations, preferably in a subject in need. In another aspect, the present invention comprises the use of an AChE-derived peptide as an agent for *ex vivo* manipulation of cells to induce granulocyte cell differentiation, wherein said peptide is denoted by SEQ. ID. NO.1.

The AChE-derived peptide denoted by SEQ. ID. NO.1 is also to be used as an agent for pre-transplant priming of hematopoietic stem cells.

In a further aspect, the present invention provides the use of an AChE-derived peptide in the preparation of a pharmaceutical composition for the treatment and/or prevention of conditions that trigger low granulocyte count, such as leukopenia, wherein said peptide is denoted by SEQ. ID. NO.1. Said composition may also be used in pre-transplant priming of hematopoietic stem cells.

In an even further aspect, the present invention provides a method of treatment of conditions that induce leukopenia, comprising the steps of administering a therapeutically effective amount of an AChE-derived peptide or a composition thereof to a subject in need, wherein said AChE-derived peptide is denoted by SEQ. ID. NO.1.

The invention also refers to a method for the prevention and/or treatment of conditions wherein lymphocyte activity is reduced, such as chronic stress, autoimmune diseases, inflammation, rheumatoid arthritis, multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), fibromyalgia, multiple chemical sensitivity, post-irradiation, chemotherapy in a subject in need, comprising administering a therapeutically effective amount of an AchE-derived peptide to an individual suffering or prone to said conditions, wherein said peptide is denoted by SEQ. ID. NO.1.

The present invention also discloses a method for detecting changes in the activity of lymphocytes, comprising measuring the expression of AChE-R on the surface of lymphocytes.

The invention provides a method of prevention and/or treatment of conditions wherein lymphocyte activity is reduced, such as chronic stress, autoimmune diseases, inflammation, rheumatoid arthritis, multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), fibromyalgia, multiple chemical sensitivity, post-irradiation, chemotherapy in a subject in need, comprising obtaining blood from said subject, isolating immature cells and contacting said

cells with an AChE-derived peptide, wherein said peptide is denoted by SEQ. ID. NO.1.

In addition, a method of priming of hematopoietic stem cells pre-transplant is presented, comprising obtaining said cells from a subject in need of granulocytes, isolating immature cells and contacting said cells with, or exposing said cells to an AChE-derived peptide, wherein said peptide is denoted by SEQ. ID. NO.1.

Lastly, the invention also provides a method of inducing adult blood cells to produce cytokines, comprising obtaining said cells from a subject in need of cytokine-producing blood cells, isolating immature cells and contacting said cells with an AChE-derived peptide, wherein said peptide is denoted by SEQ. ID. NO.1. This method is particularly advantageous for patients with neutropenia.

Brief Description of the Figures

Figure 1: Flow cytometric approach to AChE splice variants.

A. C-terminal amino acid sequence unique to the human AChE-S variant.

B. C-terminal amino acid sequence unique to the human AChE-R variant (the sequences in A and B share a similar core domain. Note that ASP, but not ARP, includes a C-terminal cysteine residue (asterisk) that enables AChE-S multimerization).

An scFv-myc tagged antibody selected against the C-terminal sequence of AChE-S from a phage display library (anti-ASP1) and a polyclonal antibody produced against synthetic ARP (drawings) enabled specific detection of each of these variants.

C. Flow cytometric sub-classification of hematopoietic cells using anti-CD45. Shown are adult peripheral blood cells divided into lymphocytes, monocytes, granulocytes and red blood cells, depending on their expression of CD45. Each dot corresponds to one cell.

D. Anti-ASP1 scFv purity was verified by gel electrophoresis. Elution from Ni-NTA column with 250 mM imidazole, revealed 30-kDa band (arrow).

Figure 2: Enzymatic AChE activity in hematopoietic blood cells.

A. Cytochemical staining reveals acetylthiocholine hydrolysis activity (brown-gray) in all cell lineages (arrows) from all three sources.

B. Counterstaining with May-Gruenwald's/Giemsa highlights the different characteristic morphologies of the smeared cells. Note gray color of cytochemically positive cells (arrows).

C. Quantitation of cell positive for AChE activity for each blood cell group and arbitrary measurement of brown intensity n=30 cells.

Abbreviations: L, lymphocytes; G, granulocytes; R, red blood cells; M, monocytes.

Figure 3: Cell surface and intracellular AChE-S and -R labeling in post-partum peripheral blood cell populations.

AChE-S and AChE-R were detected using an anti-ASP scFv antibody with a myc tag and anti-myc FITC or a polyclonal rabbit antibody and anti-rabbit FITC, respectively. Positive cells (solid line) were defined by a shift to the right as compared to the control (dashed line) histogram. This figure represents one of 15 reproducible analyses.

Abbreviations: C, Cytoplasmic; S, Surface.

Figure 4: Immunochemical analyses of cord blood cell lysates.

AChE-S and -R epitopes were detected in 0.83 mg/ml protein extracts from the three different sub-populations of cord blood.

A. Plasmon resonance traces reflected interactions of anti-ASP1 scFv antibody with extracts of granulocytes (G); lymphocytes (L); monocytes (M) and red blood cells (R). Real time interaction (x-axis) is expressed in RU (y-axis).

B. Shown are the relative contents of AChE-S and -R expressed as RU between the respective antibodies and their epitopes in the different cell lysates,

standardized to the amount of total protein in the extract (top) or the number of lysed cells (bottom).

C. Immunoblot using the phage anti-ASP1 and lysates of the noted cell populations. AChE-S and its various cleaved products are labeled. PC12 cells known to express AChE-S were used as a positive control.

Figure 5: Development- and stress-associated expression of AChE variants within blood cell populations.

Positive cell fractions were quantified by flow cytometry and divided into cells with the corresponding variants in the cytosol and on the surface of the noted cell populations. Columns present the percent of positive cells in 15 samples from each source (mean ± standard error of the mean). Nonspecific signals were subtracted. Solid and crosshatched bars represent cytoplasmic and surface expressions, respectively.

A. AChE-S

B. AChE-R

Abbreviations: R, red blood cells; G, granulocytes; M, monocytes; L, lymphocytes.

Figure 6 : Surface AChE-R on lymphocyte subpopulations.

A. Surface AChE-R was detected in T and B lymphocyte sub-populations on all three sources analyzed. Shown are cells from adult peripheral blood. Upper panel is surface AChE-R expression on T cells and lower expression on CD19+CD45+ B lymphocyte. Background staining (dashed curve) and surface AChE-R staining (bold curve). Insert: B cells were defined by their high expression of the pan-B marker CD19 (y-axis) and CD45 (x-axis). T cells were labeled with the pan-T marker CD3 together with CD45.

B. Surface AChE-R contents on lymphocyte sub-populations. An average of ten samples from each source of cells were used. Shown are mean percent values of cells expressing surface AChE-R, and the mean fluorescent intensity (MFI) ± standard error of the mean. Significant differences values (t-test, $p<0.05$) are marked by an asterisk.

Abbreviations: C, cord blood; A, adult peripheral blood; P, post-delivery peripheral blood.

Figure 7: Spatiotemporal shifts in embryonic AChE mRNAs within blood cell forming tissues.

A. Schematic of the human *ACHE* gene and its alternative mRNAs. The core of human AChE is encoded by three exons, and parts of additional regions encode the variant-specific C-terminal sequences. Transcription begins at E1, and E2 encodes a leader sequence that does not appear in any mature protein. In addition to a proximal promoter (red line adjacent to E1), a distal enhancer region (the other red line) is rich in potential regulatory sequences, some of which are shown as wedges.

B. Sagital section of a human embryo showing the hematopoietic organs - AGM (aorta-gonad-mesonephros, blue), LIV (liver; green), SPL (spleen; red), and BM (bone marrow; brown).

C. Scheme of gestational shifts in hematopoietic processes shows the relative levels of blood cell formation in the various hematopoietic organs throughout human gestation [Tavassoli, M. (1991) *Blood Cells* 17:269-281]. Ages for which *in situ* hybridization was performed are marked by gray columns.

D. Representative *in situ* hybridization in liver micrographs from human fetuses at the noted gestational ages. Selective probes for each of the alternative human AChE mRNA transcripts showed increased expression (red precipitate) of AChE-R mRNA at 16 weeks of gestation, at the same time when the liver changes from erythropoiesis to myelopoiesis.

E. Line colors representing (as in C) spatiotemporal changes in labeling intensity and standard error of the mean (SEM) for each probe and organ, expressed as percentage of red pixels in each slide [Grisaru (1999a) *id ibid.*]. Note that AChE mRNA expression increases parallel to active hematopoiesis in the examined organs (N = 4-6 tests for each organ in each gestational age). mRNA peaks in the liver at 16 weeks, coinciding with a shift in fetal liver hematopoiesis, from erythropoiesis to myelopoiesis [Porcellini, A. *et al.* (1983) *Int. J. Cell Cloning,* **1**: 92-104].

Figure 8: Post-partum blood profile.

Shown are blood profile changes that occurred in women following their delivery day (black columns; DD) and three days post-partum (white columns; PP). Normal blood count ranges are represented by grey shaded areas.

A. Hemoglobin level (Hgb) decreased, platelet (Plat) counts increased and leukocyte (WBC) counts increased during the period studied.

B. It is noteworthy that the sustained leukocytosis was due to a sustained elevated granulocyte (Gran) count, while the monocyte (Mono) and lymphocyte (Lymph) counts remained in the normal range. Asterisks indicate statistically significant differences between DD and PP (n = 23 patients).

C. In vivo model for stress.

(C1) Plasma cortisol levels in newborns (NB), non-pregnant adult women (Ct) and post-partum mothers (PP) after delivery. Note high levels of cortisol in mothers after normal delivery, reflecting stress.

(C2) Cortisol levels in mothers' serum correlate with the leukocytosis found post-partum.

(C3) Selective AChE-R accumulation in granulocytes following stress. Presented are the fractions of granulocytes positive for AChE-R in the blood of post-partum mothers (representing stress condition), compared with newborns and non-pregnant adult women controls. Note that AChE-R accumulates in mature granulocytes under elevated cortisol conditions.

(C4) AChE-R expression under stress. Plasma cortisol levels in mothers correlate with AChE-R presence in granulocytes, but not in monocytes or lymphocytes. Asterisks mark statistically significant differences from control values.

D. Stress induced AChE-R in white blood cells correlates with presence of catalytically active AChE in the plasma. The graph shows a direct correlation between AChE-R expression in all types of white blood cells and AChE activity in the plasma of post-partum mothers: granulocytes, monocytes and lymphocytes.

Figure 9: Stress induced AChE-R positive granulocytosis.

A. Plasma cortisol levels in newborns (NB), non-pregnant women (Ct) and mothers after delivery (PP). Note high levels of cortisol in mothers after normal delivery, reflecting a stressful event.

B. Cortisol levels in mothers' serum correlate with the leukocytosis found post-partum.

C. Selective AChE-R accumulation in granulocytes following stress. Presented are the fractions of granulocytes positive for AChE-R in the blood of post-partum mothers (representing stress condition), compared with newborns and non-pregnant women (representing non-stress conditions). Note that AChE-R accumulates in mature granulocytes following stress.

D. AChE-R expression under stress: Plasma cortisol levels in mothers correlate with AChE-R presence in granulocytes but not in monocytes or lymphocytes. This may further explain the granulocytosis following stress [Abo and Kawamura (2002) *id ibid.*].

Figure 10: Stress induced AChE-R in white blood cells correlates with presence of active AChE-R in the plasma.

The figure shows a direct correlation between AChE-R expression in all types of white blood cells and its activity in the plasma of post-partum mothers.

A. Granulocytes.

B. Monocytes.

C. Lymphocytes.

Figure 11: ARP_{26} operates as an inducer of ACHE gene expression.

A. Folding pattern of human AChE [Soreq and Seidman (2001) *id ibid.*] with the cleavable ARP C-terminal domain, the structure of which is not yet known, highlighted.

B. Average labeling densities for 10-20 individual human cord blood CD34+ cells treated for 24 hours with the noted doses of ARP_{26} in the absence of other growth factors and subjected to *in situ* hybridization with probes selective for each of the alternatively spliced variants of AChE mRNA. Note the

concomitant increases in all transcripts, peaking at 2 nM ARP_{26}, and the uniform nature of this response in all of the analyzed cells.

C. AChE-R is present in the plasma of post-partum mothers in higher amounts than in the plasma from non-pregnant adult women, and smaller C-terminal fragments are correspondingly elevated in the post-partum plasma.

D. Shown are representative micrographs of the cells from panel B. The lower part of the panel shows the cytochemical staining for AChE catalytic activity in the presence of 10^{-5} M iso-OMPA, selective inhibitor of butyrylcholinesterase and nuclear staining with DAPI. Note the increased appearance of brown precipitates of AChE activity mainly under 2 nM of ARP_{26}, indicating increased endogenous AChE activity.

Figure 12: ARP_{26} potentiates myeloid expansion *ex vivo*.

Flow cytometric analysis of CD34+ derived hematopoietic cells was performed after 2 weeks in liquid culture. Each analysis included 10,000 events and regions were set according to the appropriate isotype controls to exclude nonspecific labeling (first line). The percentage of immature stem cells (left column), committed myeloid (middle column) and mature myeloid cells (right column) that developed in the presence of each supplement is indicated by numbers on the relevant dot plots. Unlabeled cells appear as black dots, double labeled, as green dots. Note similar patterns under the influence of cortisol and ARP_{26}, but not ASP_{40} and PBAN.

Figure 13: Cytokine elevation under increased ARP effects.

A. Cytokine levels, in pg/mL were determined in the plasma of post-partum and adult control women and in newborns by flow cytometry using the human inflammation cytometric bead array kit (BD Bioscience) in a particle based immunoassay. Note elevation of IL-12, IL-6, and IL-1β under post-partum conditions. n=15 in each group.

B. The proposed concept involves stress-induced elevation of plasma cortisol, which promotes AChE-R overproduction in peripheral mononuclear cells. C-terminal cleavage of AChE-R yields ARP, which amplifies AChE-R

overproduction independently of cortisol. AChE-R's accumulation potentiates ACh hydrolysis, which alleviates the nicotinic α7 AChE control over pro-inflammatory cytokine production, resulting in elevated TNFα, IL-6 and IL1β.
C. To explore the potentially causal relationship between elevated AChE-R production in mononuclear cells and cytokine plasma levels, adult peripheral mononuclear cells were incubated for 24 hours with or without 2nM ARP_{26}. Note the significant increase of IL-6, IL-10 and TNFα levels, but not the anti-inflammatory cytokine IL-12, (fluorescence intensity) in the presence of ARP26.

Figure 14: Priming of CD34+ hematopoietic stem cells enhances their post-transplantation engraftment.
The graph shows, in percentage, FACS analysis of CD34+ cells engraftment in mouse bone barrow four weeks post-transplant. Cells incubated with ARP (squares) clearly engrafted better, compared to control (diamonds). The triangles in the graph represent cells incubated with 2nM of ASP, the C-terminal peptide of AChE-S, as another control.

Detailed Description of the Invention
The inventors have demonstrated that overproduction and C-terminal cleavage of the stress-induced AChE-R isoform induced long-lasting granulocytosis, independently of cortisol levels.

Thus, it is an object of the present invention to provide the use of an AChE-R-derived peptide as an inducer of granulocytopoiesis. Yet another object of the invention is to provide methods and compositions for the prevention and/or treatment of conditions leading to low white blood cell count in general, and leukopenia in specific. In addition, such treatments may increase cytokine production in patients who have lost the capacity to induce the same in response to external stimuli. These include, for example, aged patients in whom cytokine levels cannot be induced anymore because their cholinergic

control over such cell population is desensitized. These and other objects of the present invention will become apparent as the description proceeds.

In this view, in a first aspect, the present invention provides the use of an AChE-derived peptide as an agent for the induction of the production of granulocytes, wherein said peptide is denoted by SEQ. ID. NO.1. The peptide used by the invention comprises the following amino acid sequence:

N' – GMQGPAGSGWEEGSGSPPGVTPLFSP – C'

Said peptide is also denoted herein as ARP, or ARP_{26}.

Any functional derivatives and functional fragments of the above-defined peptide may be used in the invention. The terms functional derivatives and functional fragments used herein mean the peptide, or any fragment thereof, with any insertions, deletions, substitutions and modifications, which is capable of inducing granulocyte cell differentiation and/or cytokine production.

The inventors have demonstrated, in the following Examples, how peripheral cholinergic stress responses, in particular overproduction and C-terminal cleavage of the stress-induced AChE-R variant, resulted in long-lasting granulocytosis, likely independent of elevated cortisol levels.

Combining the *in vitro* results presented in Table 2, with the *in vivo* findings, specially of Figures 7, 8 and 12, lead the inventors to provide, in the present invention, the use of an AChE-derived peptide as an agent for the induction of the production of granulocytes in a subject in need, wherein said peptide is denoted by SEQ. ID. NO.1. Said peptide may also be an agent for the induction of repopulation and/or rematuration of granulocytic cell populations, preferably in a subject in need.

Interestingly, the inventors have shown that, during human fetal development, AChE-R mRNA expression was observed only in the developing liver for a limited time window (Fig. 7). The transient increase in AChE-R mRNA paralleled the period of fetal liver myelopoiesis, supporting the notion that AChE-R is physiologically relevant for *in vivo* myelopoiesis.

In another aspect, the present invention comprises the use of an AChE-derived peptide as an agent for *ex vivo* manipulation of cells to induce granulocyte cell differentiation, wherein said peptide is denoted by SEQ. ID. NO.1.

The AChE-derived peptide denoted by SEQ. ID. NO.1 is also to be used as an agent for pre-transplant priming of hematopoietic stem cells.

The presence of a functional glucocorticoid response element in the upstream *ACHE* promoter [Grisaru *et al.* (2001) *id ibid.*], combined with the transient post-partum increase in serum cortisol [Mastorakos, G. and Ilias, I. (2000) *Ann. NY Acad. Sci.* **900**: 95-106] could explain the initial transcriptional enhancement of *ACHE* gene expression in hematopoietic cells. However, the transient nature of cortisol elevation also implies that a different transcriptional enhancing signal(s) should extend this response after the first few hours. That ARP_{26} by itself elevated *ACHE* gene expression in CD34+ progenitors provided a tentative explanation for this prolonged induction, suggesting that the overproduced cleavable AChE-R can regulate its own production. ASP_{40}, the C-terminal peptide of AChE-S, failed to induce such effects (Fig. 12), supporting the specificity of the effect of ARP on prolonged granulocytosis. *Vis-à-vis* the results obtained in Example 12, ARP may be used to treat hematopoietic stem cells *ex vivo*, driving the cells to the granulocytic differentiation pathway.

The positive correlation between AChE-R contents in myeloid cells, and AChE hydrolytic activities in the post-partum serum, points to nucleated blood cells as being the source of soluble blood AChE. The effects exerted by AChE-R on

the proliferation and maturation of granulocytes could then be due to both the catalytic and the non-catalytic properties of AChE-R and its cleavable C-terminal peptide, which corresponds to ARP. At the catalytic level, AChE-R excess should lead to reduced ACh concentrations in the post-partum serum. The existence of nicotinic [Wang, H. (2003) *id ibid.*] and muscarinic [Hellstrom-Lindahl, E. and Nordberg, A. (1996) *J. Neuroimmunol.* **68**: 139-144; Mita, Y. *et al.* (1996) *Eur. J. Pharmacol.* **297**: 121-127] ACh receptors on myeloid cells suggests reduced cholinergic input to those cells under stress. This, in turn, would alleviate the control over production of pro-inflammatory cytokines, increasing their concentration and inducing further proliferative and cell activation signals [Borovikova (2000) *id ibid.*; Tracey (2002) *id ibid.*; Wang (2003) *id ibid.*]. The present study thus adds AChE-R production as a preliminary step followed by the reduced action of ACh as an anti-inflammatory regulator, potentially explaining prolonged post-stress granulocytosis.

At the non-catalytic level, the present findings suggest the induction of signal transduction processes by the C-terminal peptide cleaved from AChE-R. When injected into the mouse brain, fluorescently labeled ARP_{26} accumulated in the cytoplasm of nerve cells. Thus it seems that the ARP_{26} peptide belongs to that class of peptides with self-cell penetrance capacity [Nijholt *et al.* (2003) *Mol. Psych.* **Oct 28** (Epub ahead of print)]. A two-hybrid screen pointed to PKCβII and its scaffold protein RACK1 as AChE-R binding partners [Birikh, K.R. *et al.* (2003) *Proc. Natl. Acad. Sci. USA* **100**: 283-288]. The reported involvement of PKC signaling in myeloid cell activation [Bassini, A. *et al.* (1999) *Blood*, **93**, 1178-1188; Kawashiima, K. and Fujii, T. (2000) *Pharmacol. Ther.* **86**: 29-48] potentially implicates this interaction in the maturation and/or activation of granulocytes in the post-partum blood.

In a further aspect, the present invention provides the use of an AChE-derived peptide in the preparation of a pharmaceutical composition for the treatment and/or prevention of conditions that trigger low granulocyte count, such as

leukopenia, wherein said peptide is denoted by SEQ. ID. NO.1. Said composition may also be used in pre-transplant priming of hematopoietic stem cells.

The preparation of pharmaceutical compositions is well known in the art and has been described in many articles and textbooks, see e.g., Gennaro A. R. ed. (1990) *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Easton, Pennsylvania, and especially pages 1521-1712 therein.

The inventors found development and trauma-associated changes in *ACHE* gene expression *in vivo* as follows. AChE-S also appeared in the cytoplasmic compartment of blood cells, whereas AChE-R, rarely expressed in adult brain neurons, occurred in a significant fraction of all blood cells. Under stress, there was an increase in the fraction of AChE-S presenting myeloid cells, unlike muscle or nerve cells where its expression is not stress-modified [Brenner (2003) *id ibid.*; Meshorer (2002) *id ibid.*]. Lastly, the AChE-R variant, previously reported to be soluble in CD34+ cord blood cells, was also associated with the surface of mature peripheral blood (PB) cells.

Blood cell inflammatory and immune processes involve a finely tuned balance between myeloid cell activation, proliferation and differentiation. In this respect, increased AChE-S production, previously reported to reflect apoptosis [Zhang *et al.* (2002) *Cell Death Differ.* **9**: 790-800] may serve to prevent uncontrolled expansion of those cells that respond to the proliferation signals of AChE-R and/or its cleavable C-terminal peptide ARP [Grisaru (2001) *id ibid.*]. Reduced AChE-R densities on the cell surface of B lymphocytes under stress should further increase the chances of ACh to activate these cells by interacting with their ACh receptors [Wang (2003) *id ibid.*]. The development and stress-induced changes in *ACHE* gene expression of myeloid cells are hence likely to facilitate the hematopoietic responses to external stimuli.

Myeloid cell proliferation, another stress-associated process, may be expected to increase under these conditions due to the larger concentration of AChE-R in positive cells [Grisaru (2001) *id ibid.*]. It would be interesting to test whether this process involves protein kinase C signaling reported as being associated with myelopoiesis [Kovanen, P. E. *et al.* (2000) *Blood* **95**:1626-32], which would be compatible with AChE-R's capacity to form multi-protein complexes with PKCβII in brain neurons [Birikh (2003) *id ibid.*].

In an even further aspect, the present invention provides a method of treatment of conditions that induce leukopenia, comprising the steps of administering a therapeutically effective amount of an AchE-derived peptide or a composition thereof to a subject in need, wherein said AchE-derived peptide is denoted by SEQ. ID. NO.1.

Thus, the invention also refers to a method for the prevention and/or treatment of conditions wherein lymphocyte activity is reduced, such as chronic stress, autoimmune diseases, inflammation, rheumatoid arthritis, multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), fibromyalgia, multiple chemical sensitivity, post-irradiation, chemotherapy in a subject in need, comprising administering a therapeutically effective amount of an AchE-derived peptide to an individual suffering or prone to said conditions, wherein said peptide is denoted by SEQ. ID. NO.1.

In addition, the invention refers to a method for inducing a shift in the activity of lymphocytes *in vitro* or *ex vivo*, comprising contacting an AChE-derived peptide with lymphocytes for a suitable period of time.

As shown in Example 5, B lymphocytes in post-delivery mothers lose most of their surface AChE-R, but maintain high levels of cytoplasmic AChE-R expression, an unprecedented response pattern unique to these cells. This result suggests that the changing pattern of AChE molecules in lymphocytes might reflect a change in lymphocytic activity in response to variations in

cholinergic stimuli, under stress situations. Thus, the AChE peptide might be a potent regulator of lymphocytes activity, *in vivo*, *ex vivo* and *in vitro*.

Therefore, the present invention also discloses a method for detecting changes in the activity of lymphocytes, comprising measuring the expression of AChE-R on the surface of lymphocytes.

The invention provides a method of prevention and/or treatment of conditions wherein lymphocyte activity is reduced, such as chronic stress, autoimmune diseases, inflammation, rheumatoid arthritis, multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), fibromyalgia, multiple chemical sensitivity, post-irradiation, chemotherapy in a subject in need, comprising obtaining blood from said subject, isolating immature cells and contacting said cells with an AChE-derived peptide, wherein said peptide is denoted by SEQ. ID. NO.1.

In addition, a method of priming of hematopoietic stem cells pre-transplant is presented, comprising obtaining said cells from a subject in need of granulocytes, isolating immature cells and contacting said cells with, or exposing said cells to an AChE-derived peptide, wherein said peptide is denoted by SEQ. ID. NO.1.

Lastly, the invention also provides a method of inducing adult blood cells to produce cytokines, comprising obtaining said cells from a subject in need of cytokine-producing blood cells, isolating immature cells and contacting said cells with an AChE-derived peptide, wherein said peptide is denoted by SEQ. ID. NO.1. This method is particularly advantageous for patients with neutropenia.

Disclosed and described, it is to be understood that this invention is not limited to the particular examples, process steps, and materials disclosed herein as such process steps and materials may vary somewhat. It is also to be

understood that the terminology used herein is used for the purpose of describing particular embodiments only and not intended to be limiting since the scope of the present invention will be limited only by the appended claims and equivalents thereof.

It must be noted that, as used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The following Examples are representative of techniques employed by the inventors in carrying out aspects of the present invention. It should be appreciated that while these techniques are exemplary of preferred embodiments for the practice of the invention, those of skill in the art, in light of the present disclosure, will recognize that numerous modifications can be made without departing from the spirit and intended scope of the invention.

Examples

Experimental Procedures

Tissue and cell preparations:

- Cells were retrieved from umbilical cords of newborns of uncomplicated full-term pregnancies, as described [Grisaru (2001) *id ibid.*], in anti-coagulant citrate dextrose solution formula A-supplemented bags (Baxter, Deerfield, IL).
- Peripheral blood from adult healthy women and from mothers within the first 24 hours post-delivery was obtained from discarded samples of routine blood counts.

- Paraffin-embedded sections from electively aborted normal human embryos were prepared as previously described [Grisaru (1999b) *id ibid.*].
- Peripheral mononuclear and CD34+ cells were enriched to 85% by separation on gelatin and Ficol-Hypaque gradients followed by CD34 immune magnetic beads (Dynal, Great Neck, NY), essentially as described [Grisaru (2001) *id ibid.*; Pick, M. *et al.* (1998) *Br. J. Haematol.* **103**:639-650].

The use of human material in this study was approved by the Tel Aviv Sourasky Medical Center Ethics Committee according to the regulations of the Helsinki accords.

Variant-specific antibodies:

Monoclonal human antibody fragments were selected from a phage display library, using ASP, a synthetic peptide with the C-terminal sequence unique to human AChE-S, as target for selection. The 90% pure anti-ASP1 antibody was obtained as soluble single-chain Fv (scFv) including a myc tag and a His6 tail [Flores-Flores, C. *et al.* (2002) *J. Neural Transm.* **62(suppl)**:165-179]. Polyclonal affinity-purified rabbit antibodies directed towards the C-terminal sequence unique to human AChE-R (ARP) were obtained after repeated rabbit challenges with a glutathione *S*-transferase-ARP fusion protein (Fig. 1) [Sternfeld, M. *et al.* (2000) *id ibid.*].

Detecting AChE variants

$2x10^6$ cells (50 µl) were incubated (30 minutes, 4°C) with anti-CD45-PerCP (20 µl, 0.4 µg; BD Bioscience, San Jose, CA) and scFv purified anti-ASP1 (5 µl, 1 µg) or rabbit anti-human polyclonal anti-ARP (5 µl, 1.4 µg), washed with 15 ml 1% BSA in phosphate-buffered saline (PBS), and centrifuged (600 x *g*, 5 minutes, 4°C). Secondary antibodies were added to 50 µl of resuspended cells (30 minutes, 4°C), anti-c-myc FITC for detecting anti-ASP (5 µl, 1 µg; Caltag, Burlingame, CA) or anti-rabbit-FITC for detecting anti-ARP (3 µl, 3 µg; Zymed, San Francisco, CA). Cells were washed as above, and red blood cells were lysed with 1 ml of 1:10 diluted FACS lysis buffer (BD Bioscience; 12

minutes, 4°C). Non-specific staining was evaluated by incubating with FITC-labeled secondary antibodies and anti-CD45-PerCP only. Surface AChE-R expression was detected on lymphocytes by anti-CD19-APC (5 µl, 1 µg; Caltag) to identify B cells or anti-CD3-APC (5 µl, 1 µg; Caltag) to identify T cells. Intracellular proteins were detected in permeabilized cells (IntraStain, Dako, Glostrup Denmark). Cytochemical staining of catalytically active AChE was performed as previously reported [Lev-Lehman, E. *et al.* (1997) *Blood* 89:3644-53].

Flow cytometric acquisition and analysis:

Six-parameter, 4-color flow cytometry involved collection with a BD FACS Calibur (BD Bioscience, San Jose, CA) of at least 50,000 events per sample. For CD34+ cells, expanded cells were analysed by 3- and 4-color flow cytometry with PerCP conjugated anti-CD34, FITC-conjugated anti-CD15 and PE conjugated anti-CD38 or PE anti-CD33 (BD Bioscience, San Jose, CA). Relevant isotype control antibodies were used to detect non-specific background fluorescence. The total number of expanded cells for each lineage was calculated by multiplying their relative proportions by the number of viable cells in each culture. To detect AChE-R, cells were incubated with CD45-PerCP, followed by permeabilization using Intrastain Kit (Dako, Glostrup, Denmark), staining with anti-human ARP_{26} primary antibody [Kawashima, K., and T. Fujii (2000) *Pharmacol. Ther.* 86:29-48], and detection with an FITC-conjugated goat anti-rabbit Fab antibody (Jackson Laboratory, Bar Harbor, ME). Fluorescent detectors sensitivity was set and monitored with Quantum beads (Bangs Laboratories Inc., Fishers, IN). Mean Fluorescent Intensity (MIF) served as a measure of AChE-R content in analyzed cells. When multiplied by the percent fractions of AChE-R-positive cells, MFI values reflected the total content of AChE-R in the analyzed blood cell samples.

Solubilization of cellular antigens:

Cord red blood cells were isolated by centrifugation (600 x *g*, 20 minutes). The plasma and upper layer of cell sediment were removed. Leukocytes were

isolated on Ficoll-Hypaque (Pharmacia, North Peapack, NJ). Granulocytes found below the Ficoll-Hypaque layer were isolated and remaining red blood cells were lysed (BD Bioscience). Mononuclear cells found above the Ficoll-Hypaque, containing monocytes and lymphocytes were washed in 1% BSA-PBS (600 x *g*, 5 minutes, 4°C), re-suspended in 10 ml of Iscove's modified Dulbecco medium (Biological Industries, Beit Haemek, Israel) supplemented with 10% fetal calf serum (Biological Industries) and incubated (90 minutes, 37°C, 100% humidity, 5% CO_2), allowing monocytes to adhere. Non-adherent cells containing highly enriched lymphocytes were washed with 1% BSA-PBS and adherent monocytes were scraped and washed in 1% BSA-PBS. Cell populations, all above 95% pure (tested with antibodies specific for the population and flow cytometry), were washed with PBS and re-suspended in high salt detergent buffer (300 mM NaCl, 0.5% Triton X-100, 50 mM Tris HCl, pH 7.6), including the protease inhibitor cocktail Complete Mini (Roche Molecular Biochemicals, Mannheim, Germany). After 1 hour shaking at 4°C, samples were centrifuged (10 minutes, 10,000 x *g*, 4°C). Supernatants were stored at -80°C for further analysis. Protein concentration was determined using a Lowry assay kit with albumin as protein standard (Bio-Rad, Hercules, CA).

Immunoblots:

Anti-ASP1 antibody displayed on the phage surface [Flores-Flores (2002) *id ibid.*] was used. The pellet of separated cells was resuspended in 10 ml of denaturing buffer (2% SDS, 50 mM Tris HCl, pH 6.8). Soluble cell lysates (6µg of protein) were run on 4-20% polyacrylamide gels and electroblotted. Membranes were blocked (10% BSA, PBS, 0.5 % Tween 20, 18 hours, 4°C), and incubated with the phage carrying the anti-ASP1 antibody ($2.6x10^8$ transforming units/ml, blocking buffer, 2h, room temp.). Following washing (PBS-0.5% Tween20) membranes were incubated with horseradish peroxidase/anti-M13-conjugated antibody (1 hour, room temperature; Amersham Pharmacia Biotech, Little Chalfont, UK) diluted 1:10000 in 5%

BSA-PBS, 0.1% Tween20. Peroxidase activity was detected using an ECL kit from Amersham.

Surface Plasmon Resonance (SPR):

SPR measurements (BIAcore 3000 System, Uppsala, Sweden) used the anti-ASP1 scFv and anti-ARP antibodies immobilized on a CM5 sensor chip through their primary amine groups [Johnsson, B. *et al.* (1991) *Anal. Biochem.* **198**:268-77]. The matrix was activated with 70 µl of 0.4 M N-ethyl-N'-(dimethyl-aminopropyl)-carbodiimide and 0.1 M N-hydroxysuccinimide, and 200 µg/ml of the particular antibody in 10 mM sodium acetate, pH 3.5, were injected at a flow rate of 10 µl/min in 10 mM HEPES, pH 4.0, 150 mM NaCl, 3.4 mM EDTA and 0.005% polysorbate 20 to reach surface density of between 3000 to 6000 resonance units (RU). Remaining activated carboxyl groups were blocked by injecting 70 µl of 1 M ethanolamine hydrochloride. Cord blood cell extracts in high salt detergent buffer were brought to 0.83 mg protein/ml in 10 mM HEPES pH 4.0 with 150 mM NaCl, 3.4 mM EDTA, 0.005% polysorbate 20. Carboxymethyl dextran was added to avoid non-specific binding of protein to the surface matrix. 60 µl extract doses were injected through the flowcell to which the antibody was immobilized and through a reference surface (to which no antibody was immobilized) for 2 minutes. A 10 µl pulse of 2 M NaCl achieved regeneration of the antibody in the flowcell. Data management involved multi-parameter Student's t-test statistics with p values <0.05 considered significantly different.

Cell counts and serum tests:

Blood cell counts were performed using the Coulter Gen-S analyzer (Beckman Coulter, Miami, FL). Serum was separated from these samples. Serum cortisol levels were measured by electrochemiluminescence immunoassay (ECLA) (Roche, Indianapolis, IN) and analyzed by Elecsys 1010/2010 and modular analytics E170 (Roche). AChE specific activity was determined in the serum using a standard colorimetric assay of thiocholine release from acetylthiocholine [Seidman, S. *et al.* (1995) *Mol Cell Biol* **15**:2993-3002], in the

presence of 10^{-5} iso-OMPA, a selective inhibitor of BChE. Serum cytokines levels, including tumor necrosis factor alpha (TNFα), interleukins (IL) -1β, -6, -8, and -12p-70, were assessed using the human inflammation cytometric bead array kit, CBA (BD Bioscience, San Diego, CA), for detecting soluble proteins in the serum in a particle-based immunoassay (FACScalibur, BD Bioscience, San Jose, CA). Data acquisition involved BD CellQuest software and Microsoft Excel.

In situ hybridization:

In situ hybridization procedures, were performed on freshly isolated cells using 5'-biotinylated, 2'-O-methylated AChE cRNA probes complementary to 3'-alternative human *ACHE* exons as previously described [Grisaru (2001) *id ibid.*]. Labeling intensity was assessed as the percent cytoplasmic red pixels and normalized by subtraction of background signals. Confocal microscopic scans of the cells were obtained using a MRC-1024 Bio-Rad confocal microscope (Hemel Hempsted Herts., UK). ANOVA was used for statistical calculations.

Ex vivo expansion of hematopoietic progenitor cells:

Umbilical cord blood CD34+ cells were expanded in liquid cultures in the presence of ARP_{26} or ASP_{40}, synthetic peptides with the AChE-S or AChE-R C-terminal sequences [Grisaru (2001) *id ibid.*], PBAN (a negative control insect peptide) [Nijholt *et al.* (2003) *id ibid.*] or cortisol. Peptides were used at 2nM and cortisol at stress levels of 1.2 μM. Liquid cultures were initiated and maintained in 24-well tissue culture plates (1 x 10^5 cells/well in 1 ml). Cells were grown for up to 14 days at 37°C in 5% CO_2 in a fully humidified atmosphere in IMDM supplemented with 5% autologous plasma. Fresh reagents were added every 4 days. The number of viable cells was assessed by trypan blue exclusion.

Example 1

Evaluating AChE splice variants in hematopoietic cell populations

Cytochemical staining of smeared blood cell preparations revealed acetylthiocholine-hydrolysing AChE in blood cells from the newborn, adult and post-partum sources. Particularly prominent intracellular staining was observed in adult and post-partum granulocytes, whereas enzyme activity on the cell surface was clearly observed on lymphocytes, granulocytes and monocytes from adult blood, compatible with the inventors' previous findings [Lev-Lehman (1997) *id ibid.*], but only on granulocytes from post-partum mothers. Figure 2A portrays representative micrographs (one cell from 30 analyzed) of this staining, and Figure 2B includes activity staining combined with morphology.

To attribute enzyme activities to specific AChE variants and explore their surface-cytoplasmic localization, flow cytometry was used, which combines physical characteristics of these cells with specific surface antigens. CD45, a glycosylated trans-membrane phosphatase which is expressed on the membrane of granulocytes, monocytes and lymphocytes at different intensities, but not in erythrocytes [Craig, W. *et al* (1994) *Br. J. Haematol.* 88:24-30; Xu, Z. and Weiss, A. (2002) *Nat. Immunol.* 3: 764-71]. CD45 was used to identify these blood cell populations from several human sources, which included adult non-pregnant women, adult women post-partum and cord blood from their newborns.

Antibodies directed to the unique C-terminal sequences of human AChE-S [Flores-Flores (2002) *id ibid.*] and AChE-R [Sternfeld (2000) *id ibid.*] were used in conjunction with CD45 labeling to analyze the expression of the corresponding variants or fragments thereof in the sub-classified blood cell populations. Flow cytometry measurements using naive or permeabilized cells enabled distinction between cell surface and intra-cellular localization of these variants. Quantitative values were expressed as either percent positive cells (expression levels) within each population or mean fluorescence intensities of

the positive fraction, which reflected content of the corresponding variant protein in each population (see below).

In blood cells from post-partum mothers, this analysis expectedly showed very low to undetectable levels of AChE-S and -R on the surface and in the cytoplasm of red blood cells (RBCs), compatible with AChE-E being the variant that is present and active in these cells. Increased fluorescence, measured by a shift in histogram patterns compared to background, reflected the presence of substantial AChE-S and -R levels in all of the CD45+ populations. The levels of expression of the AChE-S and AChE-R variants in ost-partum peripheral blood granulocytes and monocytes higher (both on the surface and in the cytoplasm) than in lymphocytes, which showed low levels on the surface, and somewhat higher levels in the cytoplasm (Fig. 3). The surface-cytoplasmic distribution of enzymatically active AChE within blood cells thus presented lineage-specific differences that were altered both during development and following the stress of childbirth.

Example 2

Differential concentrations of AChE variants within specific blood cell types

An independent quantification of AChE variant levels within specific blood cell types was obtained using the BIAcore technology, based on measuring the interaction of proteins in cell homogenates with antibodies covalently linked to a carboxymethyl dextran matrix adherent to the surface of a gold leaf sensor [Johnsson (1991) *id ibid.*]. Increases in the refractive index of this sensor were monitored in real time as the changes in surface plasmon resonance (SPR) angle (Fig. 4A). Non-specific SPR signals obtained in the absence of antibodies were subtracted, and protein levels were standardized either to the amount of total protein or to the number of lysed cells in each preparation, irrespective of their cellular localization (Fig. 4B).

Because cord blood lysates were examined at only one antibody concentration and one lysate concentration, the BIAcore measurements could only reflect relative antigen interaction with the antibodies, but not absolute affinity values. These relative amounts of each variant were compared within specific cell types and between the four hematopoietic cell groups. When applied to newborn cord blood cell extracts, larger signals were detected for AChE-S than for -R. Decreasing concentrations (reflected in resonance units, RU/mg protein) of AChE-S occurred in the order of granulocytes > lymphocytes > monocytes > red blood cells. A different decreasing order, lymphocytes > monocytes > granulocytes > red blood cells, was calculated per cell, suggesting that the high granulocyte concentration of AChE-S reflected the high total protein content. AChE-R signals, which were generally lower, presented similar decreasing orders in both measures (lymphocytes > monocytes > granulocytes > red blood cells, Fig.4B). The BIAcore and flow cytometry methods revealed distinct R: S ratios, e.g. for granulocytes from cord blood (Fig. 3, 4), perhaps due to different properties of the two antibodies.

A third evaluation approach involved immunoblot analysis of the corresponding cell homogenates using the AChE-S specific ASP antibody displayed on phage surface (Fig. 4C). This analysis demonstrated several immunopositive protein bands in granulocytes and lymphocytes, most of which of smaller size then the predicted full protein. A single rapidly migrating band appeared in red blood cells, with fainter, similarly migrating band in myeloid cells. These bands likely reflect proteolytic breakdown products of the ACHE-S protein in blood cells. Additionally, or alternatively, the cleaved C-terminus of AChE-S [Grisaru (1991) *id* ibid.] or an immunocompatible variant could be exposed in cell homogenates but not in intact cells.

Example 3

Distinct splice variations in development and stress

Calculating the AChE-S/AChE-R distributions as percent of positive cells in each lineage revealed distinct splice variations in development and stress.

Most cell populations included significant fractions of cells positive for both cytoplasmic and surface AChE-S and –R variants, with significantly higher numbers of AChE-R expressing cells in all adult myeloid cell populations than in fetal cells (Fig. 5). The number of granulocytes expressing cytoplasmic AChE-R was significantly higher in post-partum blood ($p<0.05$), reminiscent of the increase in AChE-R seen in brain neurons under stress [Kaufer (1998) *id ibid.*; Meshorer *et al.* (2002) *id ibid.*; Soreq and Seidman (2001) *id ibid.*]. However, AChE-S, which adheres to the membranes of brain neurons [Perrier *et al.* (2002) *Neuron* **33**:275-85], was expressed on the surface and in the cytoplasm of significantly more blood cells in post-partum mothers than in non-pregnant women (Students t-test, $p<0.05$). In addition, post-partum lymphocytes displayed a paradoxical decrease in surface AChE-R. The localization of AChE-R to the cell surface, which was rather surprising in view of its hydrophilic C-terminal peptide, may reflect interaction with as yet unknown protein variant(s) [Birikh (2003) *id ibid.*].

Example 4

Surface and cytoplasmic AChE-S and AChE-R contents in peripheral blood cells

High fluorescence intensity from comparative flow cytometry confirmed the increased protein content of the AChE-R variant compared to AChE-S of all cell types and sources tested. AChE-R protein content of granulocytes, which comprise 70 to 80% of the white blood cell compartment, were significantly higher in post-partum mothers compared to cord blood cells (Table 1), reflecting a strikingly different splicing pattern for AChE pre-mRNA in fetal blood cells than in adult cells under the post-partum stimulus. Cytoplasmic increases in AChE-R content, observed as larger mean fluorescence intensity values, occurred under stress in all cell lineages. Stress-induced increases in cell surface AChE-R appeared in granulocytes and monocytes, but not in red blood cells. In lymphocytes, cell surface AChE-R increased 8-fold from newborns to adults but declined under stress (Table 1).

Table 1: AChE variant contents in blood cells.

		Mean Fluorescence Intensity (MFI) [a]			
	% of blood leukocytes[b]	AChE-S		AChE-R	
		Cytoplasmic	Surface	Cytoplasmic	Surface
RBCs					
CB	NA	4.7 ± 1.6	1.4 ± 0.1	6.9 ± 0.3	4.1 ± 0.4
APB	NA	4.8 ± 0.6	1.4 ± 0.3	5.0 ± 0.3	4.7 ± 2.1
PPB	NA	4.5 ± 0.4	4.7 ± 0.1	**18.5 ± 1.2**	5.8 ± 0.6
granulocytes					
CB	39-63	30.1 ± 15.1	19.4 ± 3.3	57.9 ± 2.7	42.8 ± 3.4
APB	43-65	11.1 ± 0.5	**6.1 ± 0.3**	32.9 ± 3.9	62.9 ± 3.5
PPB	69-82	9.1 ± 0.4	30.1 ± 0.4	**96 ± 3**	**138 ±10**
monocytes					
CB	6-12	**21.3 ± 0.1**	22.3 ± 3.9	42.7 ± 3.0	29.1 ± 1.7
APB	6-12	10.3 ± 0.3	9.5 ± 0.7	17.8 ± 1.7	27.1 ± 6.4
PPB	6-12	14.7±0.8	21.3 ± 0.7	**57.5 ± 2.6**	**72 ± 5.8**
lymphocytes					
CB	42-62	10.4±1.3	9.7 ± 1.7	16.1 ± 0.5	24 ± 2
APB	21-46	4.8±0.3	2.2±0.1	8.2±0.7	**183±11**
PPB	13-23	5.7±0.3	10.4±0.2	**25.7±1.1**	37±2

[a]Average of 15 measurements for each population expressed as mean ± standard error are presented.
[b]Proportions of white blood cells expressed by peripheral blood leukocyte populations. Significantly different MFI values as compared with the other two sources according to t-test ($p<0.05$) analysis are presented in bold face type.

Example 5

Development and stress-induced changes in lymphocytic AChE variants

Previous reports attributed lymphocytes' AChE activity to T cells and described its increases with mitogenic stimulation [Szelenyi *et al.* (1987) *Immunol. Lett.* **16**: 49-54]. Activity was also observed in the thymus [Topilko and Caillou (1985) *Blood* **66**: 891-5], but B lymphocytes displayed very low levels of AChE, which decreased with maturation [Szelenyi *et al.* (1982) *Br. J.*

Haematol. **50**: 241-5]. In our study, CD3+ T cells presented low expression of surface AChE-R in all samples while CD19+ B cells expression was significantly higher (Fig. 6A), suggesting relevance for antibody production. Due to the majority of T cells (about 9:1 to B cells, Fig. 6A, inset), their small signals contributed significantly to the total lymphocyte output. Nevertheless, CD19+ B cell fractions expressing surface AChE-R increased in adults as compared to newborn cells and post delivery preparations. Nevertheless, B cells displayed a significant increase in fluorescence intensity from newborn cord blood to adults and post-delivery blood cells, with no change between the latter two fractions (Fig. 6B). Larger lymphocyte fractions expressed surface AChE-R in separate T and B cell populations, likely due to the high background staining in the T lymphocyte fraction (Fig. 6A).

In conclusion, considerably more myeloid cells of the post-partum mothers expressed AChE-S and AChE-R than in either control women or newborns. In contrast, B lymphocytes lost their surface AChE-R with development and under stress.

Example 6

Fetal AChE-R mRNA expression coincides with myelopoiesis

The *in vivo* expression of alternative AChE mRNA transcripts (Fig. 7A) was studied by *in situ* hybridization using paraffin-embedded human fetal sections from different gestational ages (Fig. 7A). AChE mRNAs were observed in the aorta-gonad-mesonephric region (AGM), liver, spleen and bone marrow, consistent with the spatiotemporal shifts of hematopoietic embryogenesis [Tavian (2001) *id ibid.*] and the migration of fetal hematopoiesis through the various blood forming tissues (Fig. 7C). Clear changes occurred in developing liver, with distinct labeling intensities for each of the AChE mRNA transcripts at different gestational ages (Figure 1D). At 9 weeks gestation, when the liver and spleen are initiating definitive hematopoiesis, the erythrocytic AChE-E mRNA transcript was prominently displayed in the AGM, liver and spleen. Significant levels of the synaptic transcript (AChE-S mRNA) were found at

this time in the AGM region and liver, but not in spleen, while the AChE-R mRNA variant was barely detectable in all hematopoietic tissues. At 16 weeks, during accelerated myelopoiesis, AChE-S was elevated in both liver and spleen in agreement with findings of others [Chan (1998) *id ibid.*]. A decrease in AChE-E mRNA concurrent with an increase in AChE-R mRNA was observed in the liver, suggesting a splicing shift (Fig. 7E). Subsequent decreases in all AChE mRNA variants were observed until 25 weeks. These changes were concomitant with the switch from primitive hematopoiesis, which is exclusively erythrocytic, to definitive hematopoiesis of all lineages. These results suggest that AChE-R overproduction is causally associated with myelopoiesis *in vivo*.

Example 7

Sustained post-delivery granulocytosis

To explore the relevance of peripheral cholinergic responses for *in vivo* leukocytosis, hematopoietic changes in post-partum blood samples were analyzed. Decreases in hemoglobin levels and an increase of platelet counts were observed several days post-partum, compatible with psychological anxiety, pain and blood loss, factors associated with delivery (Fig. 8A). Leukocytosis involved an elevation in granulocyte, but not monocyte or lymphocyte counts (Fig. 8B). Sequential blood cell counts following delivery showed increased that the leukocyte levels remained high several days post-partum (Fig. 8A and data not shown). Therefore, this *in vivo* model was used to assess the short- and long-term effects of a limited stressful event, parturition, on leukocytosis.

Example 8

Post-delivery cortisol escalation associates with increased granulocytic AChE-R expression

Cortisol levels were predictably elevated post-partum as compared to control groups of newborns and non-pregnant women (36.6 ± 4.2 vs. 9.3 ± 5.5 and 11.4 ± 4.1 μg/dl, $p<0.001$, ANOVA; Fig. 9A). A direct correlation emerged

between serum cortisol levels and the post-partum white blood cell (WBC) counts (R=0.55, p=0.04; Fig. 9B). This was accompanied by elevated expression of AChE-R in the cytoplasm of mature WBC (p=0.009; Fig. 9C). A significant correlation between the percent of AChE-R positive granulocytes (data not shown), but not monocytes or lymphocytes and cortisol levels (R=0.72, p=0.003; Fig. 9D) was consistent with the idea that AChE-R may have a role in the prolonged post-partum granulocytosis.

Example 9

Leukocyte AChE-R contents positively associate with plasma AChE activity

Total AChE-R contents in blood cells were evaluated by multiplying the mean fluorescence intensity (MFI) per the percent of positive cells expressing AChE-R in each cell type (granulocytes, monocytes and lymphocytes) detected by flow cytometry. In each type of circulating WBC, AChE-R contents correlated with AChE activity in the post-partum plasma (for granulocytes R= 0.984, $p<0.0001$; for monocytes R=0.962, $p<0.0001$; and for lymphocytes R=0.917, $p<0.0001$; Figure 10). Plasma AChE activity levels thus reflected AChE-R production in each type of leukocyte.

Example 10

ARP_{26} enhances endogenous *ACHE* gene expression

Cortisol elevation could explain the AChE-R excess only in the first few hours post-partum, but plasma AChE activities remained higher than control for several days (data not shown). Immunoblot analysis demonstrated intense AChE-R immunostaining, as well as C-terminal cleavage of plasma AChE-R in post-partum mothers, considerably more than in matched controls (Fig. 11A), and comparable to the extent of AChE-R cleavage occurring under lipopolysaccharide exposure [Cohen (2003) *id ibid.*]. Assuming a turnover number of $1x10^4$ molecules of acetylthiocholine (ATCh) hydrolyzed/second/AChE subunit (Taylor) and assuming, based on the immunoblot analysis, that up to half of the AChE-R is C-terminally cleaved *in*

vivo, the measured specific activity of ATCh hydrolyzed in the serum of post-partum mothers predicted peptide concentration in the range of 5-30 nM. To test the potential effect of this cleavage process, ARP_{26}, a synthetic peptide having the C-terminal sequence of AChE-R [Grisaru (2001) *id ibid.*], was added to cord blood CD34+ progenitors at 0.2, 2 and 20 nM. *In situ* hybridization followed by confocal quantification of the three AChE mRNA variants revealed, 24 hours later, increased levels of all AChE mRNA transcripts and elevated cytochemically stainable activity of the AChE protein in cultured cells (Fig. 11B). The increases peaked under 2 nM ARP_{26} and occurred to a similar extent to that observed in CD34+ cells exposed to stress-associated cortisol levels [Grisaru (2001) *et al.*]. The enhanced activity under physiologically relevant concentrations of ARP reflected an increase in endogenous AChE, as the synthetic peptide has no enzymatic capacity. It also provided a possible explanation for the prolonged increases in AChE activity in post-partum sera.

Example 11

ARP_{26}, as a single cytokine, potentiates myelopoiesis in liquid cultures

The effect of ARP_{26} on the myelopoietic expansion of human CD34+ hematopoietic stem cells over a two week period was studied. Flow cytometry was employed to monitor the development of phenotypically distinct cell populations in the presence of these various agents. Peptide controls (e.g. ASP and insect peptide PBAN) were used to study the specificity of the response. Figure 12 and Table 2 present the resultant cell growth and changes in the populations that emerged from a typical CD34+ culture. Both cortisol and ARP_{26}, but neither ASP_{40} nor PBAN, increased the total number of cells. A larger fraction of committed progenitors (CD34+CD38+) emerged in the presence of cortisol at stress levels (1.2 μM) as compared to ARP_{26} (Fig. 12). However, the expansion capacity and hence the total number of CD34+CD38+ cells was considerably higher in the presence of ARP_{26} (Table 2). Increases were observed along the entire myelopoietic differentiation pathway

(CD34+CD33+, CD33+CD15- and CD33+CD15+), supporting the notion that ARP_{26} shifts hematopoiesis towards the granulocytic lineage in a cortisol-independent pathway, expending the population of mature CD33-CD15+ granulocytes (Fig. 12 and Table 2).

Table 2: The effect of various conditions on cultured CD34+ cells.
- The numbers represent actual cell counts x 10^3 of each cell type detected.

Cell type / Treatment		CD34+ CD38+	CD34+ CD33+	CD33+ CD15-	CD33+ CD15+	CD33- CD15+
	Cell expansion index[a]	Committed progenitors	Committed myeloids	Immature myeloids	Granulocytes	Mature granulocytes
Control	1.2	0.5	0.5	1.4	2.1	4.3
Cortisol (1.2 nM)	1.6	11.0	10.6	13.0	28.5	15.4
ARP_{26} (2 nM)	11.4	29.1	31.9	94.6	64.4	220.6
ASP (2 nM)	2.0	2.5	0.4	0.5	1.9	4.2
PBAN (2 nM)	2.1	0	0.1	0.2	0.9	1.8

[a]Expansion index is a ratio of the number of viable cell/ml culture at day 14 divided by the number of cells seeded (50,000).

Example 12

AChE-R supports anti-inflammatory cytokine release from mononuclear cells

The elevated AChE-R contents and AChE activity in post-partum blood further predicted reduced ACh levels, and hence suppressed cholinergic control over the production by peripheral blood cells of pro-inflammatory cytokines [Tracey (2002) *id ibid*; Wang, H. (2003) *id ibid.*]. The levels of several inflammation/stress-associated cytokines in the plasma of post-partum mothers were hence compared to those of newborns and non-pregnant women. Elevation was observed for IL-1, -6 and TNF, both known to have pro-inflammatory and hematopoietic roles (Fig. 13A). To examine whether this increase could be causally related to AChE-R overexpression in peripheral

white blood cells, $2.5x10^6$ mononuclear cells per mL from adult women controls were incubated with 2 nM ARP (Fig. 13B). Cytokine levels in the supernatants from these cell cultures revealed, 24 hours later, a significant increase in the secretion of IL-6, IL-1 and TNF, but no change in the release of the anti-inflammatory IL-8 from cells incubated with ARP, as compared to control cells (Fig. 13C). Thus, post-partum AChE-R overexpression in peripheral nucleated blood cells could be causally associated with selective elevation of pro-inflammatory cytokines.

Example 13

Pre-transplantation priming with ARP

The AChE-derived peptide is beneficial as an agent for improving engraftment of human stem cells into bone marrow following transplantation in immune deficient mice. This is performed by priming of hematopoietic stem cells with the peptide prior to their transplantation.

NOD/SCID mice were treated with sub-lethal irradiation of 325 rad. 24 hours later, 10^5 CD34+ cells from human cord blood, treated with 2nM of ARP 2 hours prior transplantation, were introduced via i.v. 6 weeks later, CD45+ cells were analyzed by FACS. The results show that the percentage of CD45+ (human cells) found engrafted in the mice was up to 58%, whereas in the control it was up to 35% (Fig. 14).

These results show that the ARP peptide was effective in priming pre-transplant cells, and consequently improving their engrafting potential.

SEQUENCE LISTING

<110> Yissum Research Development Co. of the Hebrew University of Jerusalem
Sourasky Tel Aviv Medical Center

<120> ARP as an inducer of granulocytopoiesis, uses and methods thereof

<130> 16557/ph/03

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Pro Pro Gly Val Thr Pro Leu Phe Ser Pro
20 25

Claims

1. Use of an AChE-derived peptide as an agent for the induction of the production of granulocytes, wherein said peptide is denoted by SEQ. ID. NO.1.

2. Use of an AChE-derived peptide as an agent for the induction of the production of granulocytes in a subject in need, wherein said peptide is denoted by SEQ. ID. NO.1.

3. Use of an AChE-derived peptide as an agent for the induction of repopulation and/or rematuration of granulocytic cell populations, wherein said peptide is denoted by SEQ. ID. NO.1.

4. Use of an AChE-derived peptide as an agent for the induction of repopulation and/or rematuration of granulocytic cell populations in a subject in need, wherein said peptide is denoted by SEQ. ID. NO.1.

5. Use of an AChE-derived peptide as an agent for *ex-vivo* manipulation of cells to induce granulocyte cell differentiation, wherein said peptide is denoted by SEQ. ID. NO.1.

6. Use of an AChE-derived peptide as an agent for pre-transplant priming of hematopoietic stem cells, wherein said peptide is denoted by SEQ. ID. NO.1.

7. Use of an AChE-derived peptide in the preparation of a pharmaceutical composition for the treatment and/or prevention of conditions that trigger low granulocyte count, wherein said peptide is denoted by SEQ. ID. NO.1.

8. Use of an AChE-derived peptide in the preparation of a pharmaceutical composition for the treatment of leukopenia, wherein said peptide is denoted by SEQ. ID. NO.1.

9. Use of an AChE-derived peptide in the preparation of a composition for use in pre-transplant priming of hematopoietic stem cells, wherein said peptide is denoted by SEQ. ID. NO.1.

10. Method of treatment of conditions that trigger low cell count of granulocytes, comprising the steps of administering a therapeutically effective amount of an AChE-derived peptide or a composition thereof to a subject in need.

11. The method as defined in claim 8, wherein said AChE-derived peptide is denoted by SEQ. ID. NO.1.

12. Method of treatment of conditions that trigger low cell count of leukocytes, comprising the steps of administering a therapeutically effective amount of an AChE-derived peptide or a composition thereof to a subject in need.

13. The method as defined in claim 10, wherein said AChE-derived peptide is denoted by SEQ. ID. NO.1.

14. Method for the prevention and/or treatment of conditions wherein lymphocyte activity is reduced, such as chronic stress, autoimmune diseases, inflammation, rheumatoid arthritis, multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), fibromyalgia, multiple chemical sensitivity, post-irradiation, chemotherapy in a subject in need, comprising administering a therapeutically effective amount of an AChE-derived peptide to an individual suffering or prone to said conditions, wherein said peptide is denoted by SEQ. ID. NO.1.

15. Method for inducing a shift in the activity of lymphocytes *in vitro* or *ex vivo*, comprising contacting an AChE-derived peptide with lymphocytes for a suitable period of time.

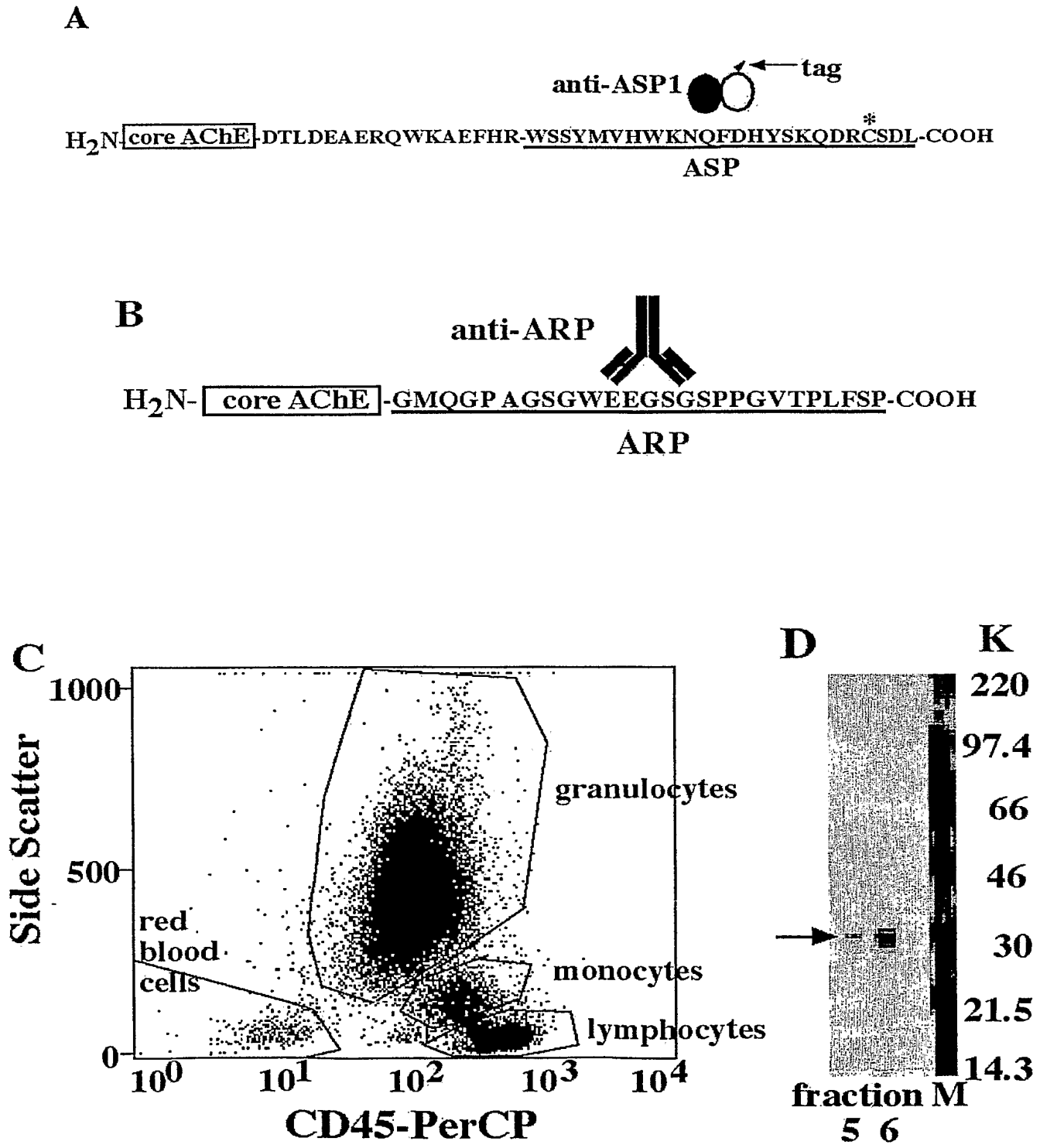
16. Method for detecting changes in the activity of lymphocytes, comprising measuring the expression of AChE-R on the surface of lymphocytes.

17. Method of treatment of conditions wherein lymphocyte activity is reduced, such as chronic stress, autoimmune diseases, inflammation, rheumatoid arthritis, multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), fibromyalgia, multiple chemical sensitivity, post-irradiation, chemotherapy in a subject in need, comprising obtaining blood from said subject, isolating immature cells and contacting said cells with an AChE-derived peptide, wherein said peptide is denoted by SEQ. ID. NO.1.

18. Method of priming of hematopoietic stem cells pre-transplant, comprising obtaining said cells from a subject in need of granulocytes, isolating immature cells and exposing said cells to an AChE-derived peptide, wherein said peptide is denoted by SEQ. ID. NO.1.

19. Method of inducing blood cells to produce cytokines, comprising obtaining said cells from a subject in need of cytokine-producing blood cells, isolating immature cells and contacting said cells with an AChE-derived peptide, wherein said peptide is denoted by SEQ. ID. NO.1.

לוצאטו את לוצאטו
LUZZATTO & LUZZATTO
By: Shalom Luzzatto עו"ד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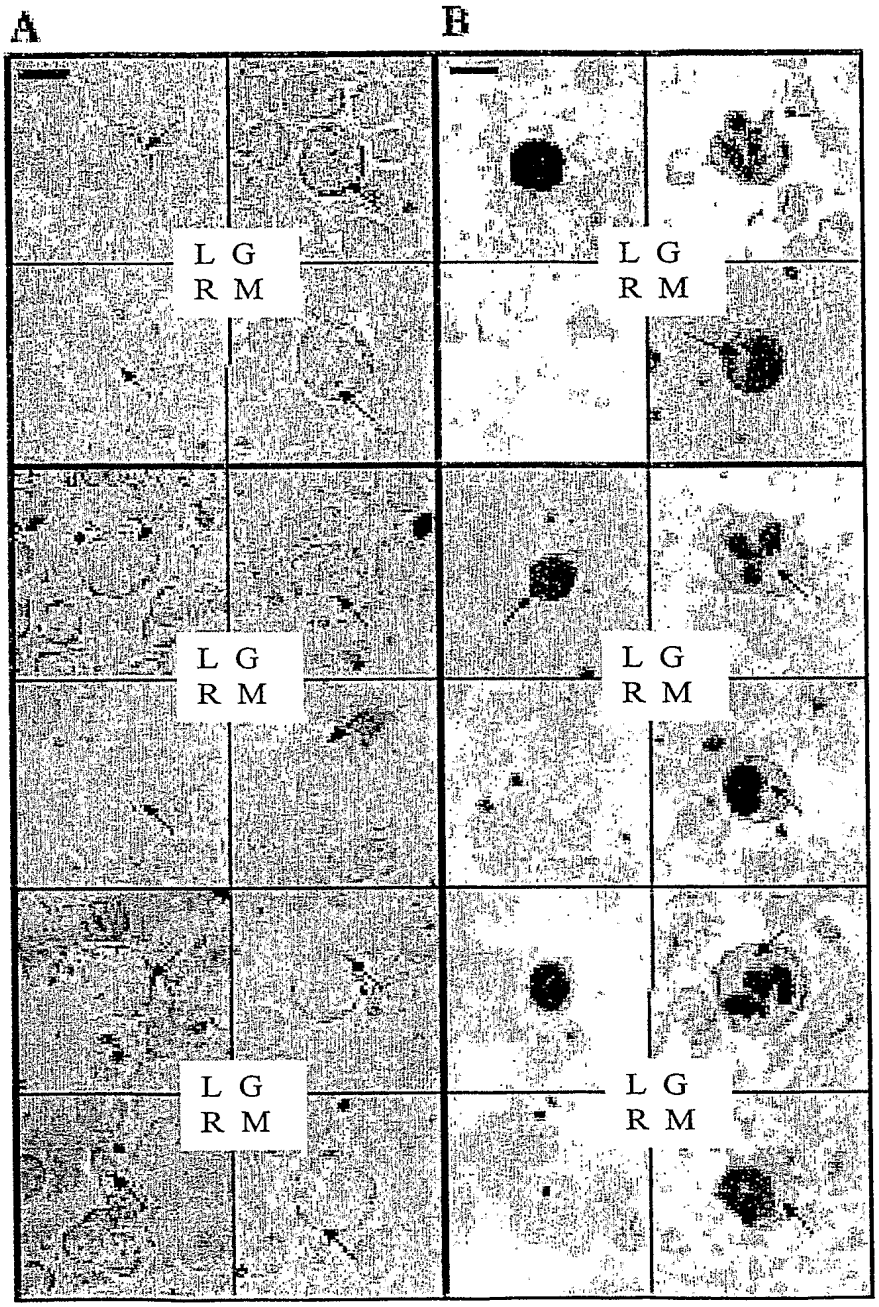
*
ASP
B
anti-ARP
H2N- core AChE -GMQGP AGSGWEEGSGSPPGVTPLFSP-COOH
ARP
C
1000
500
0
Side Scatter
red blood cells
granulocytes
monocytes
lymphocytes
10^0
10^1
10^2
10^3
10^4
CD45-PerCP
D
K
220
97.4
66
46
30
21.5
14.3
fraction M
5 6

Fig. 1



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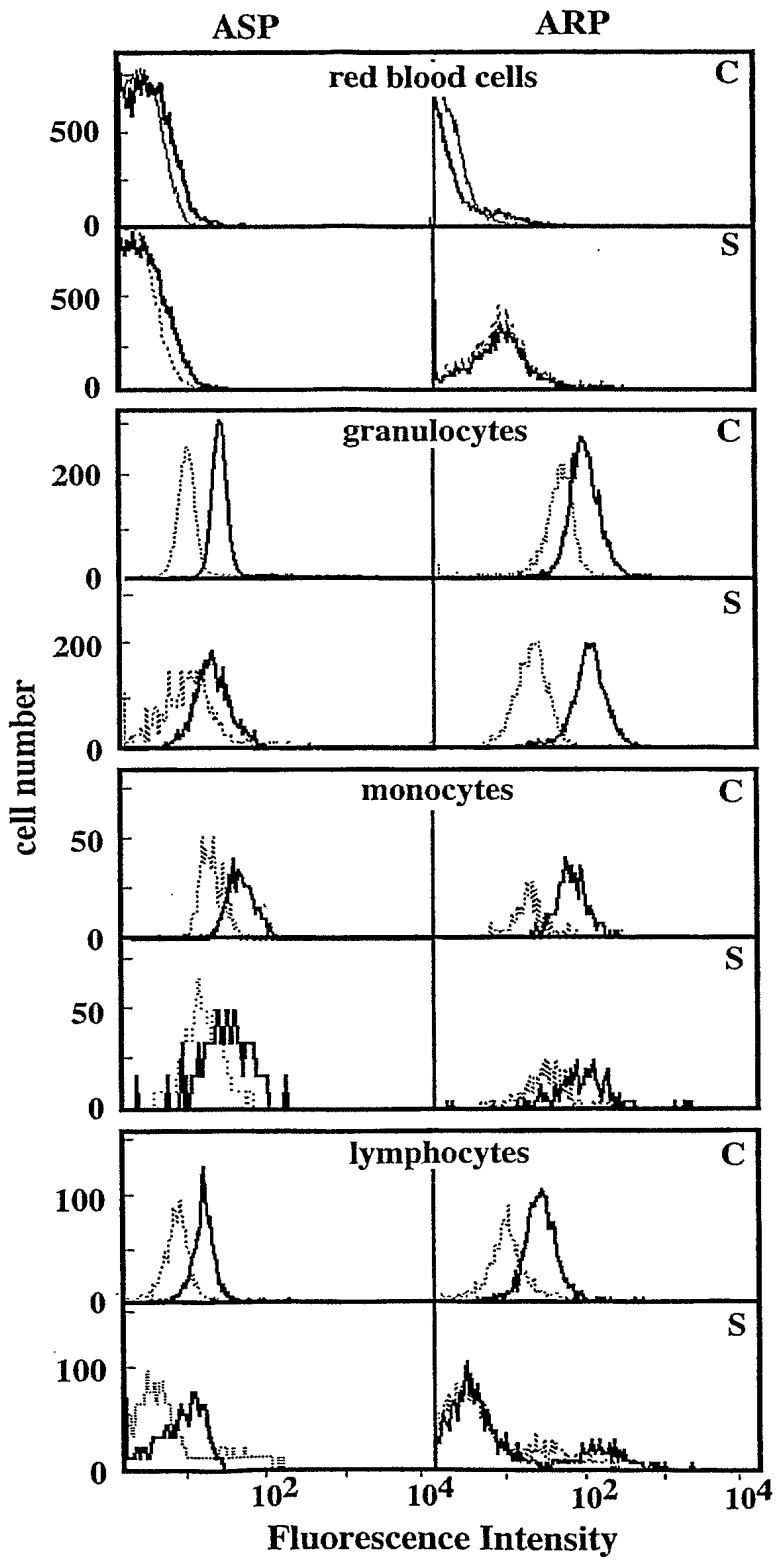
	intensity	+	++	+	+++
AB	% positive	60	60	42	100
	intensity	++	++	++	+
PPB	% positive	70	67	12	100
	intensity	+++	++	++	+

Fig. 2


ASP
ARP
red blood cells
C
S
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0
granulocytes
200
monocytes
50
lymphocytes
100
cell number
10^2
10^4
Fluorescence Intensity

Fig. 3

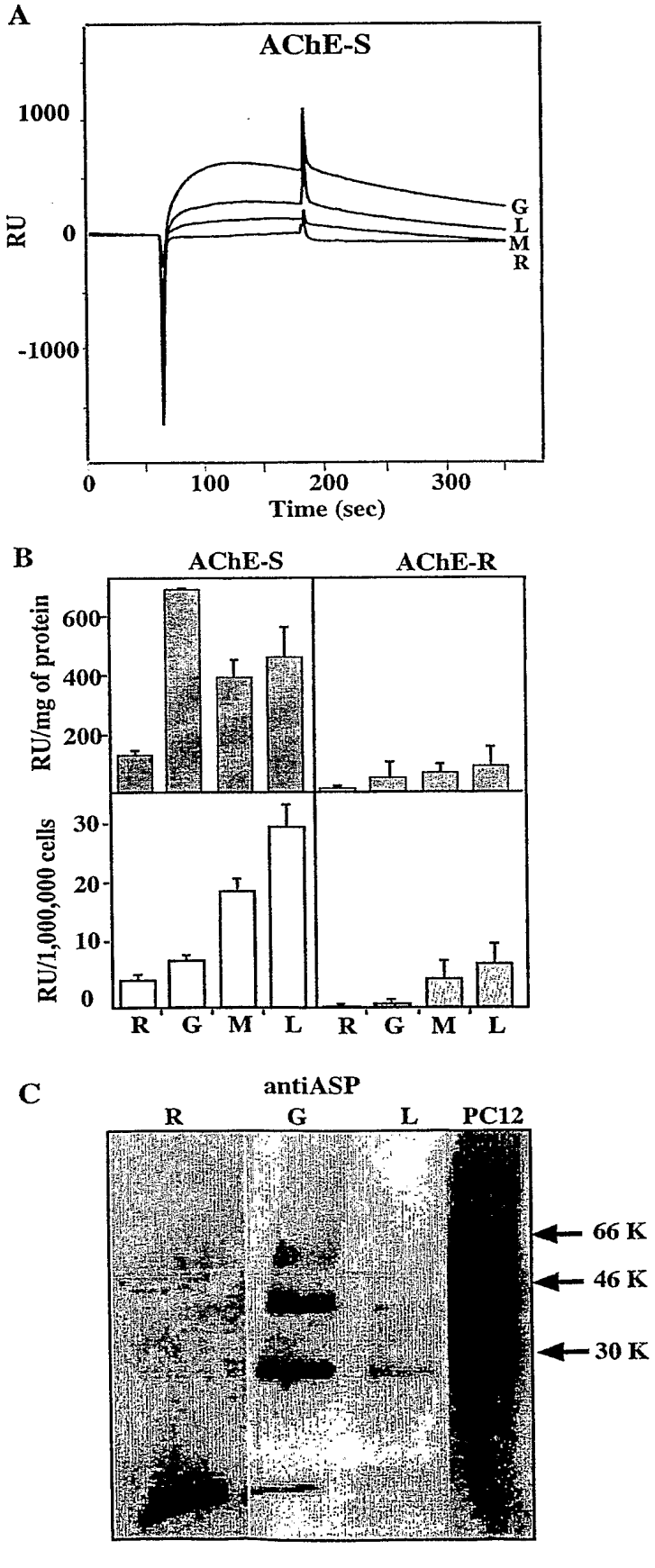
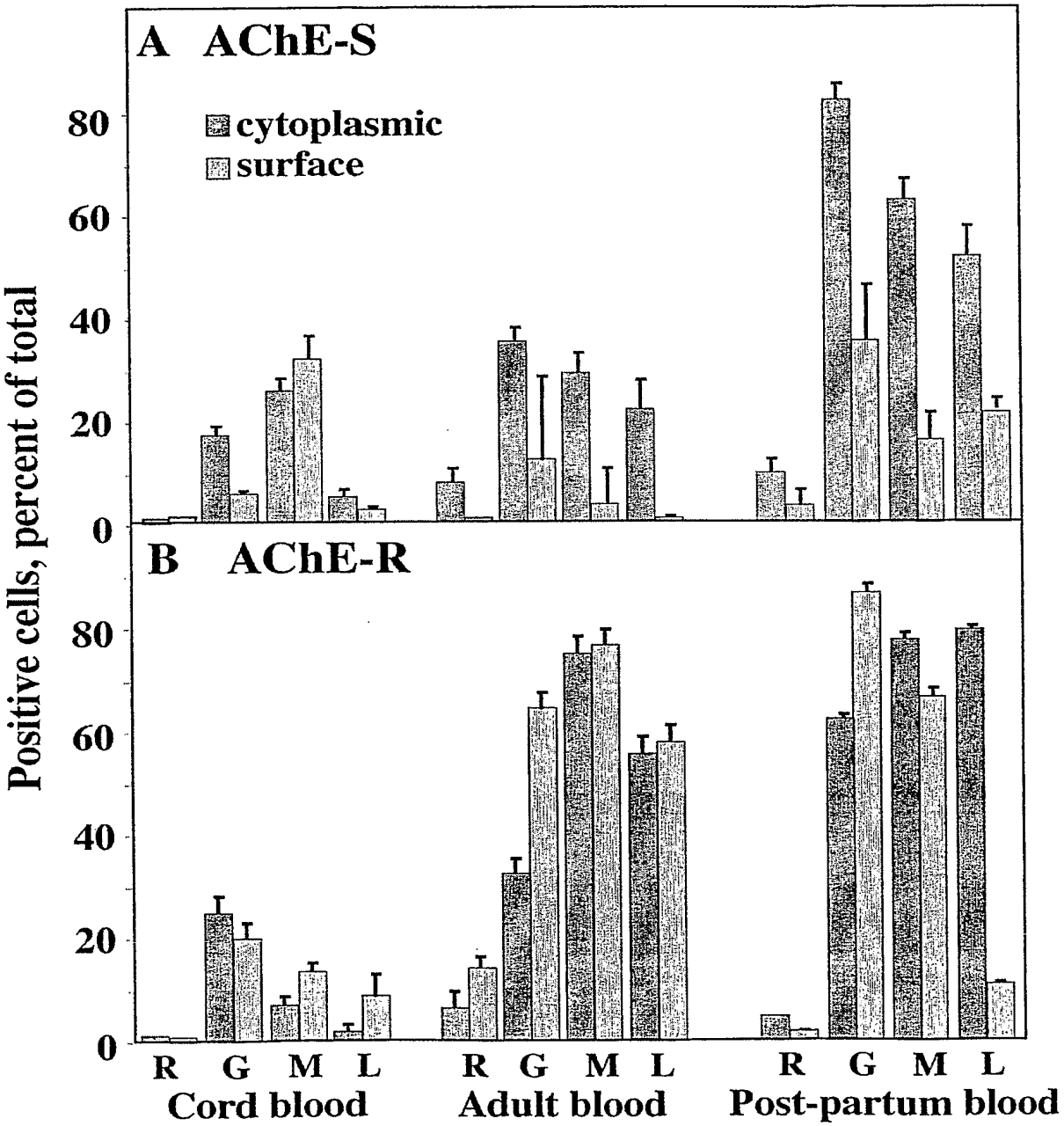

A
AChE-S
1000
RU
0
-1000
G
L
M
R
0
100
200
300
Time (sec)
B
AChE-S
AChE-R
RU/mg of protein
600
400
200
RU/1,000,000 cells
30
20
10
0
R G M L R G M L
C
antiASP
R
G
L
PC12
66 K
46 K
30 K

Fig. 4


A AChE-S
cytoplasmic
surface
B AChE-R
Positive cells, percent of total
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20
40
60
80
R G M L
Cord blood
Adult blood
Post-partum blood

Fig. 5

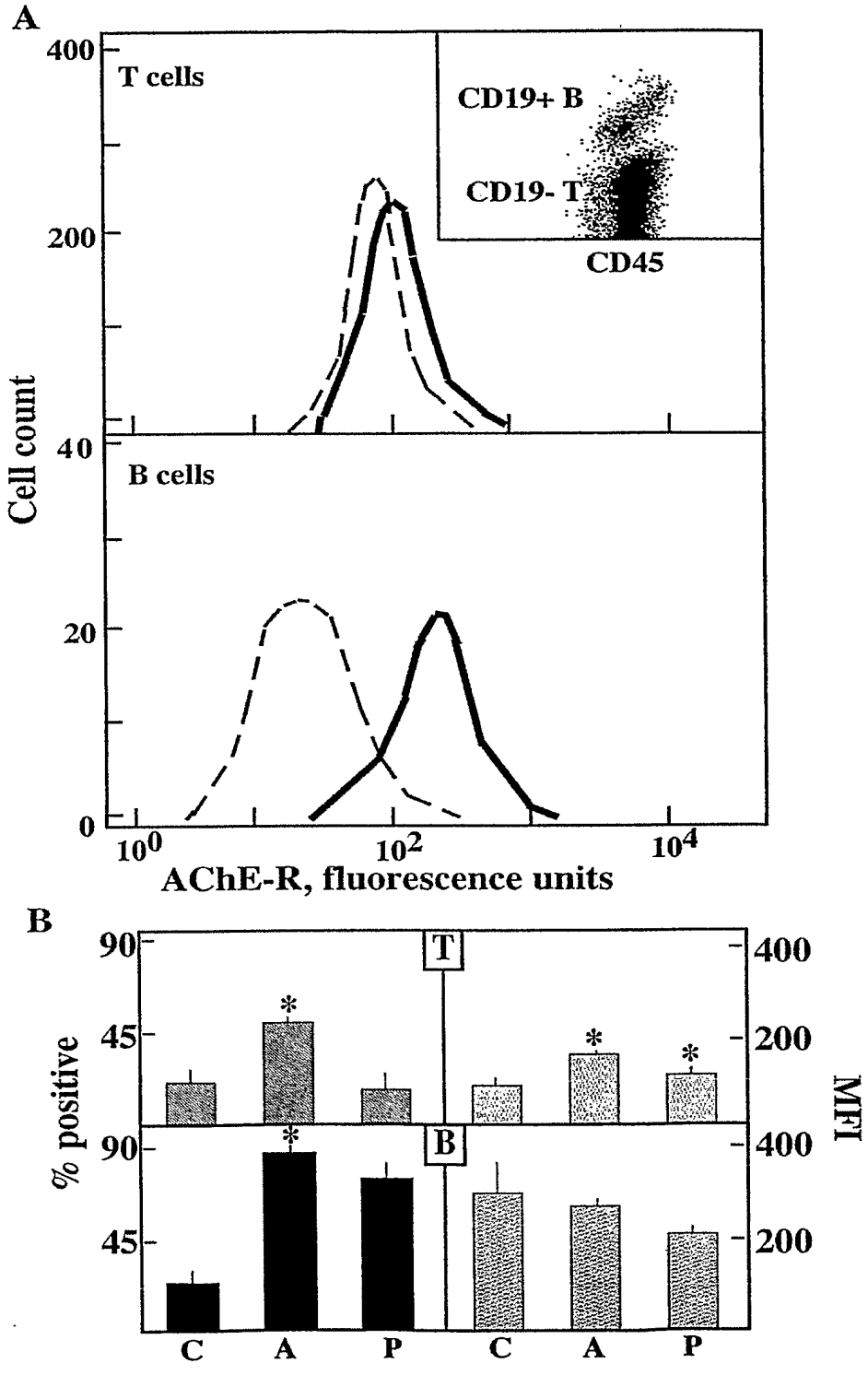
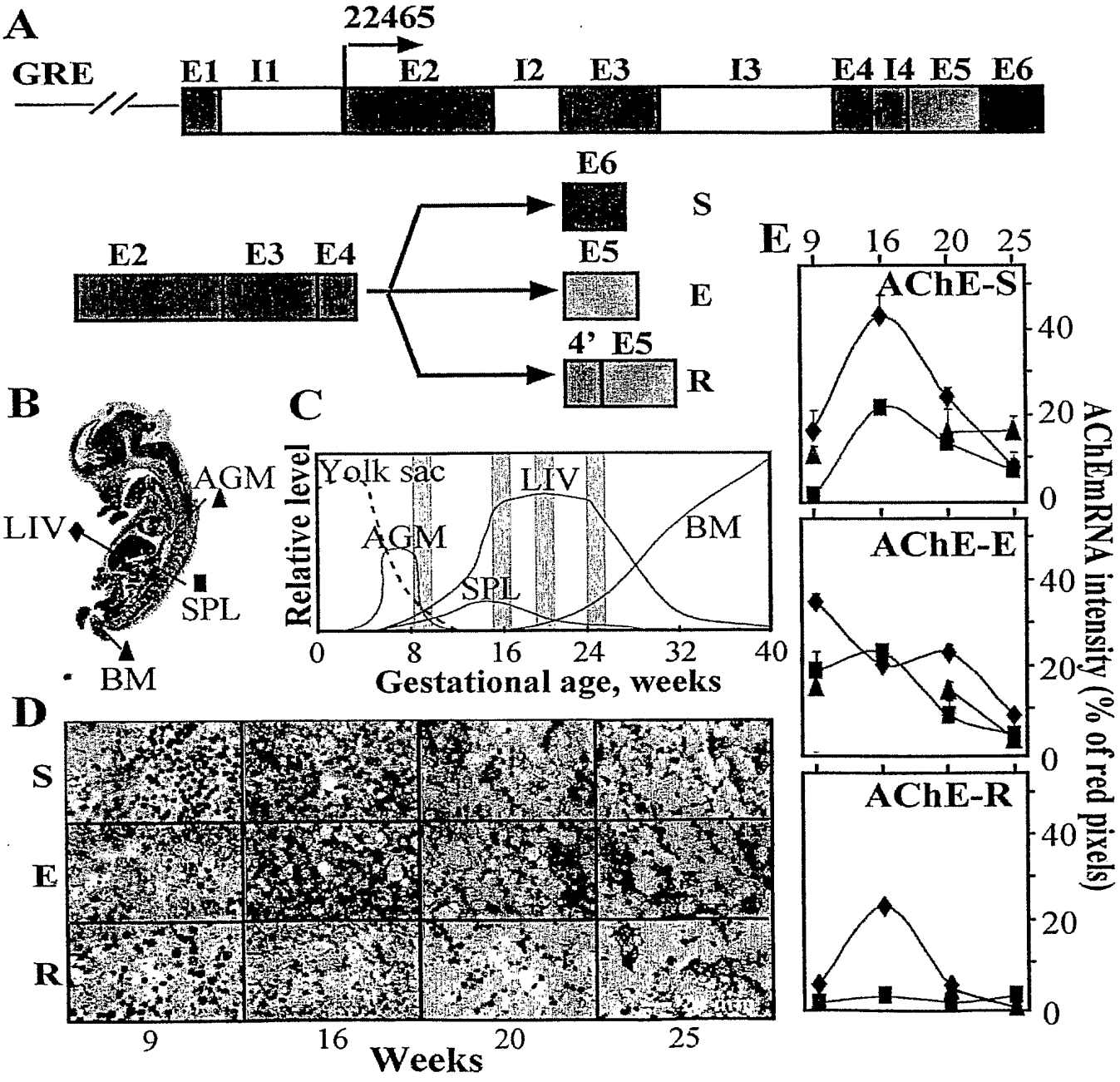

A
400
T cells
CD19+ B
CD19- T
CD45
200
Cell count
40
B cells
20
0
10^0
10^2
10^4
AChE-R, fluorescence units
B
90
T
400
45
200
% positive
MFI
90
B
400
45
200
C
A
P
C
A
P

Fig. 6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GRE
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I2
E3
I3
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S
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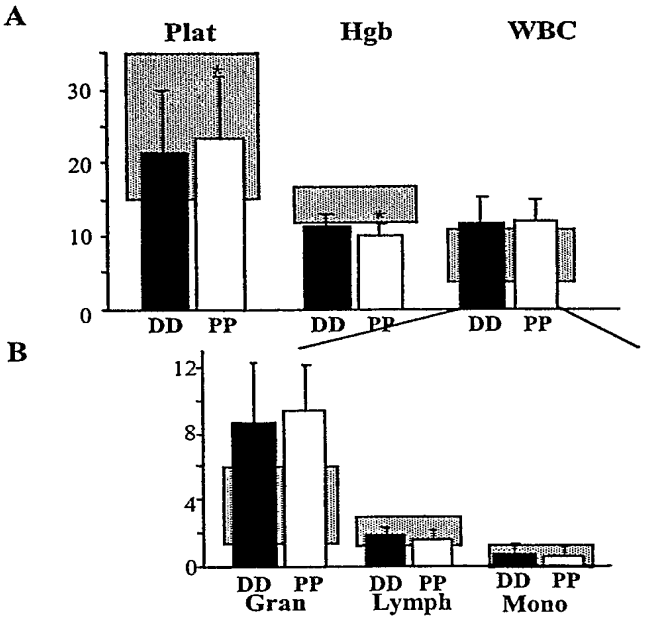
E
4' E5
R
B
AGM
LIV
SPL
BM
C
Yolk sac
LIV
AGM
BM
SPL
Relative level
0 8 16 24 32 40
Gestational age, weeks
D
S
E
R
9 16 20 25
Weeks
E
9 16 20 25
AChE-S
AChE-E
AChE-R
0 20 40
AChEmRNA intensity (% of red pixels)

Fig. 7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Plat
Hgb
WBC
30
20
10
0
DD PP
DD PP
DD PP
B
12
8
4
0
DD PP
Gran
DD PP
Lymph
DD PP
Mono
C
1
2
3
4
40
30
20
10
15
12
9
6
3
70
50
30
10
R=0.55
R=0.72
NB Ct PP
10 20 30 40 50
Cortisol, mg/dL
D
Granulocytes
Monocytes
Lymphocytes
50
40
30
20
10
0
R=0.984
R=0.962
R=0.917
40 80
20 60
5 15 25
MFI x %AChE-R

Fig. 8

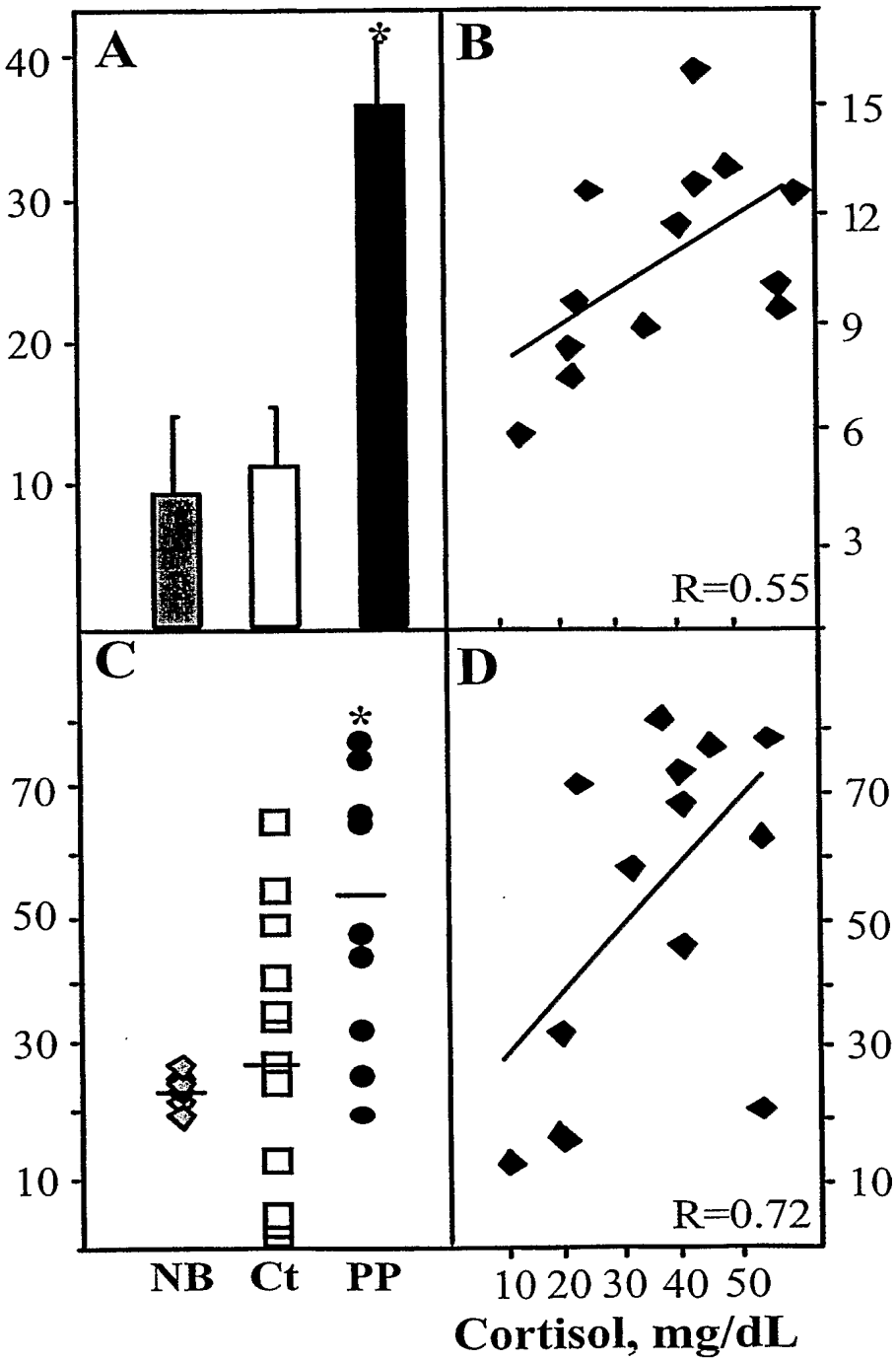
A
B
C
D
40
30
20
10
15
12
9
6
3
R=0.55
70
50
30
10
R=0.72
NB
Ct
PP
10 20 30 40 50
Cortisol, mg/dL

Fig. 9

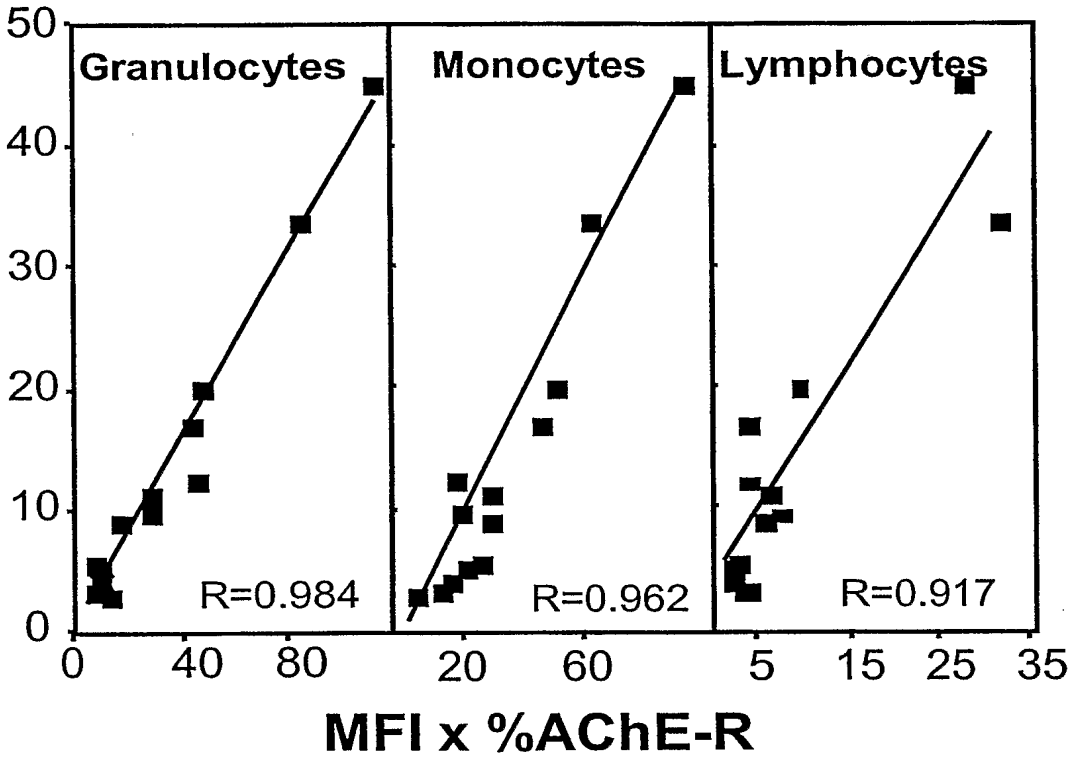
Granulocytes
Monocytes
Lymphocytes
R=0.984
R=0.962
R=0.917
50
40
30
20
10
0
0
40
80
20
60
5
15
25
35
MFI x %AChE-R

Fig. 10

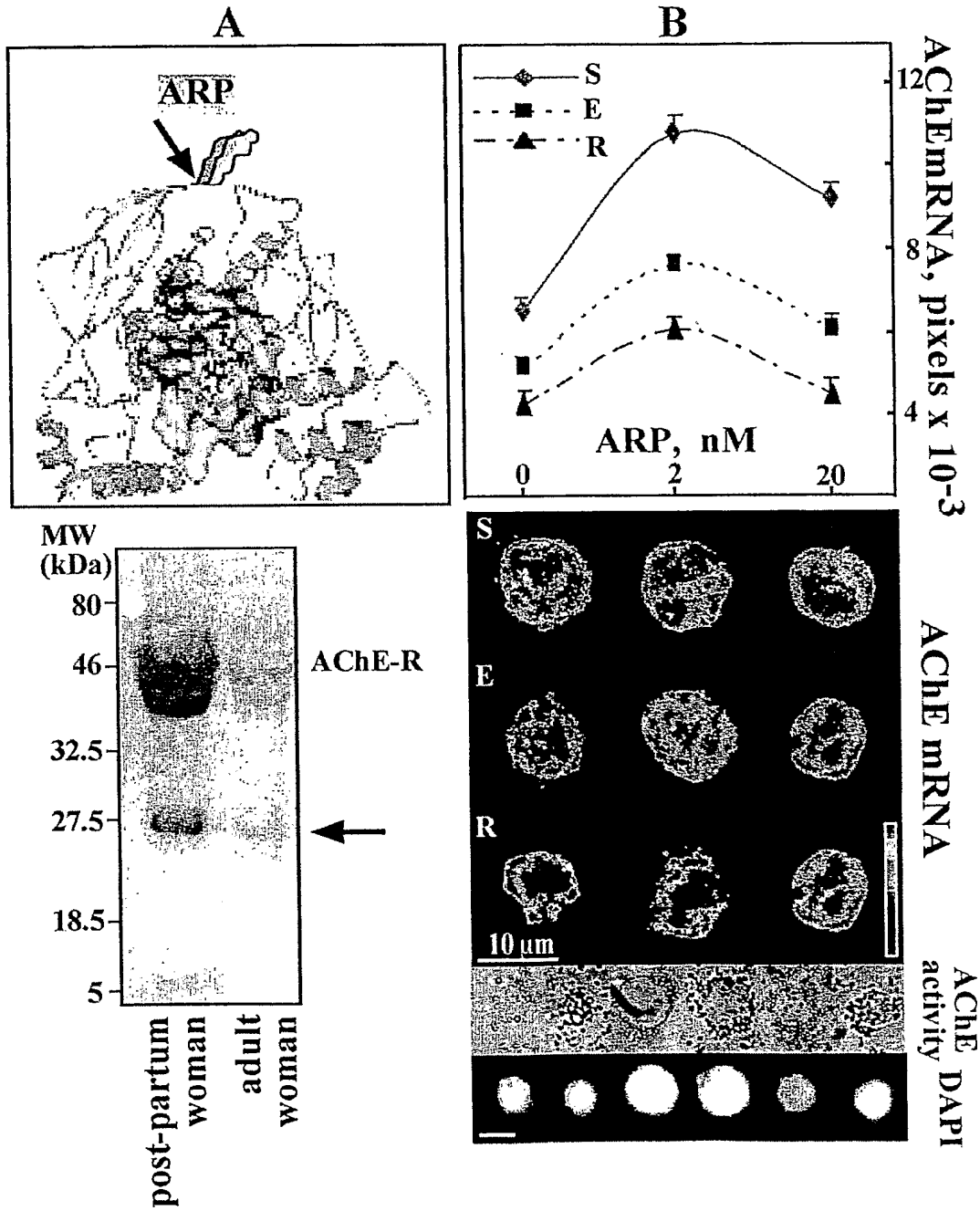
A
ARP
B
S
E
R
12
8
4
AChEmRNA, pixels x 10-3
ARP, nM
0
2
20
MW
(kDa)
80
46
32.5
27.5
18.5
5
AChE-R
post-partum woman
adult woman
S
E
R
10 µm
AChE mRNA
AChE activity
DAPI

Fig. 11

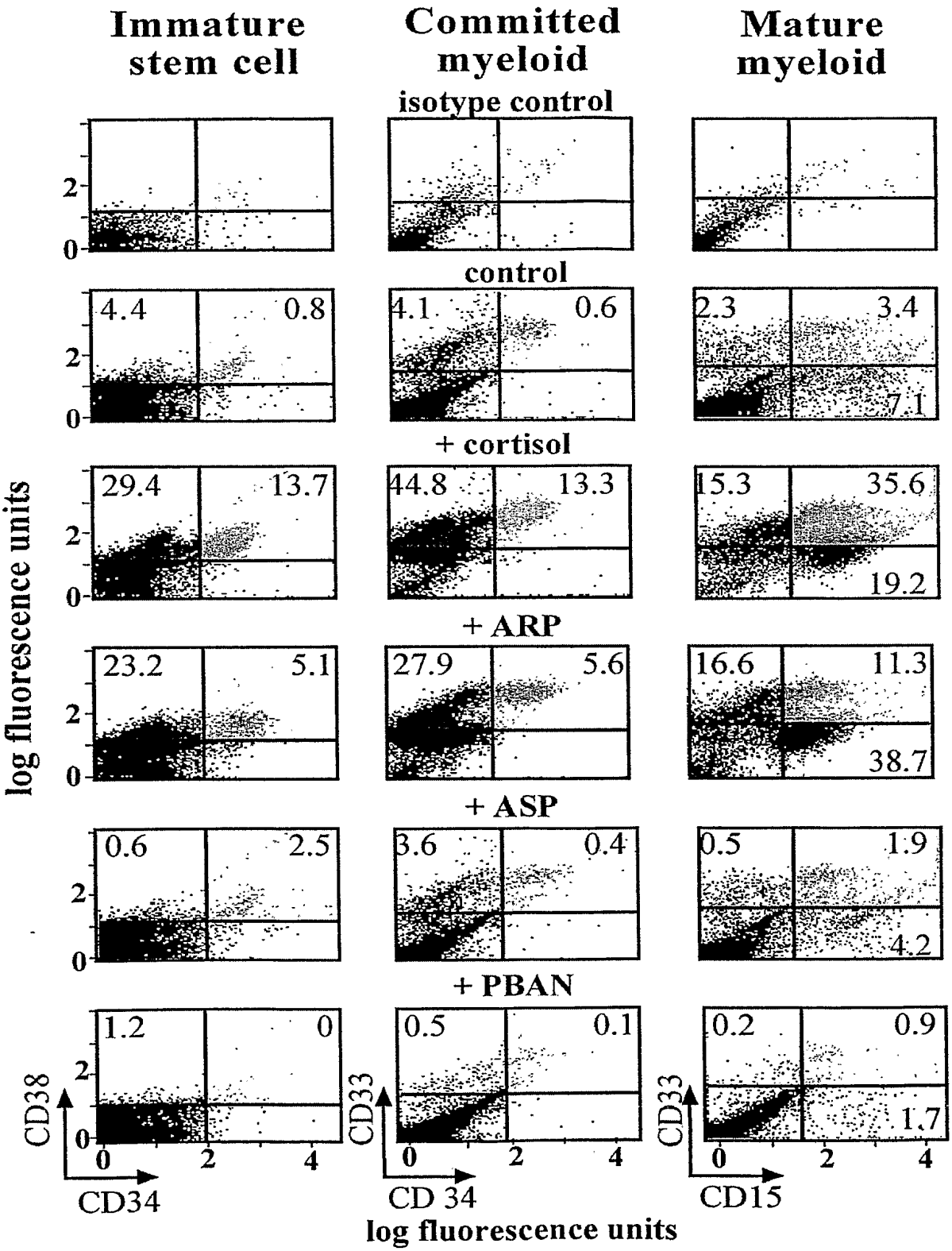
Immature stem cell
Committed myeloid
Mature myeloid
isotype control
control
+ cortisol
+ ARP
+ ASP
+ PBAN
4.4
0.8
4.1
0.6
2.3
3.4
7.1
29.4
13.7
44.8
13.3
15.3
35.6
19.2
23.2
5.1
27.9
5.6
16.6
11.3
38.7
0.6
2.5
3.6
0.4
0.5
1.9
4.2
1.2
0
0.5
0.1
0.2
0.9
1.7
log fluorescence units
CD38
CD34
CD33
CD 34
CD33
CD15
log fluorescence units

Fig. 12

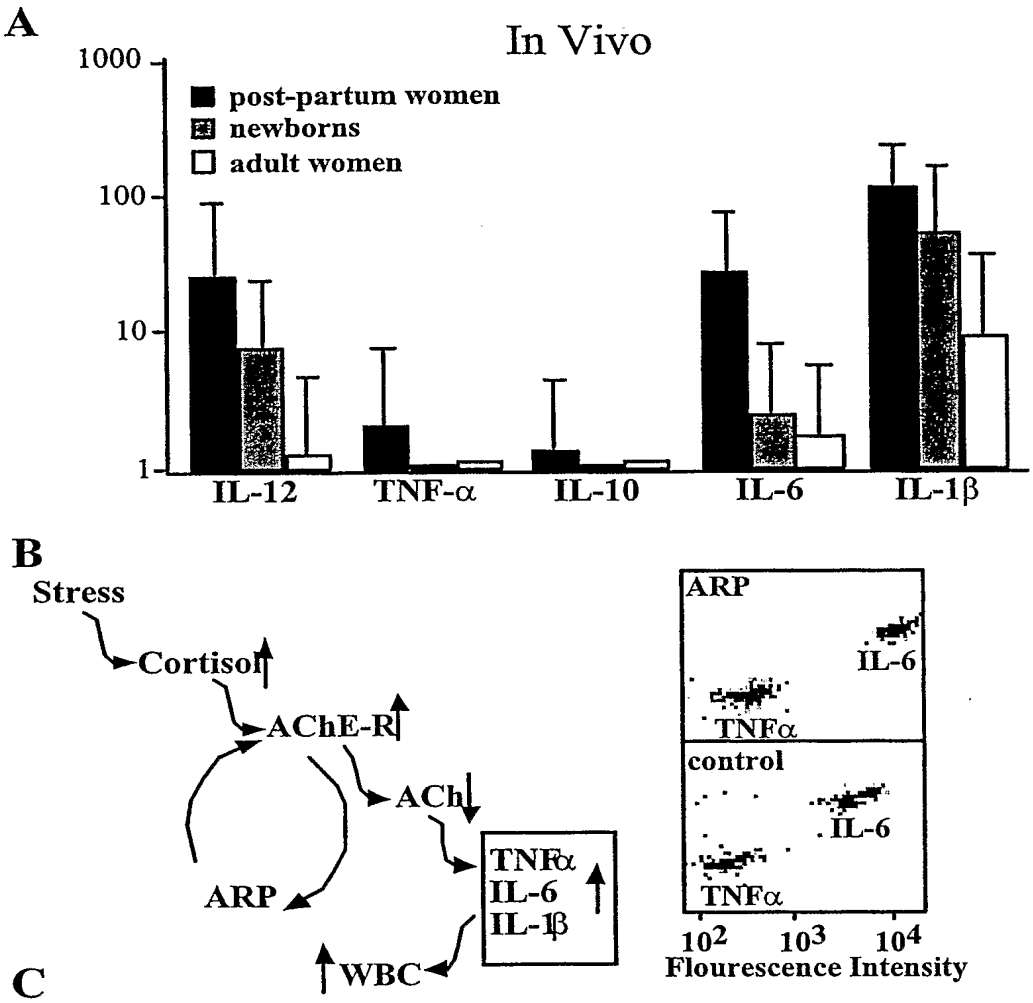
A
In Vivo
1000
100
10
1
post-partum women
newborns
adult women
IL-12
TNF-α
IL-10
IL-6
IL-1β
B
Stress
Cortisol
AChE-R
ACh
ARP
TNFα
IL-6
IL-1β
WBC
ARP
IL-6
TNFα
control
IL-6
TNFα
10²
10³
10⁴
Flourescence Intensity
C
In Vitro
10000
1000
100
10
1
ARP
control
*
IL-12
TNF-α
IL-10
IL-6
IL-1β

Fig. 13

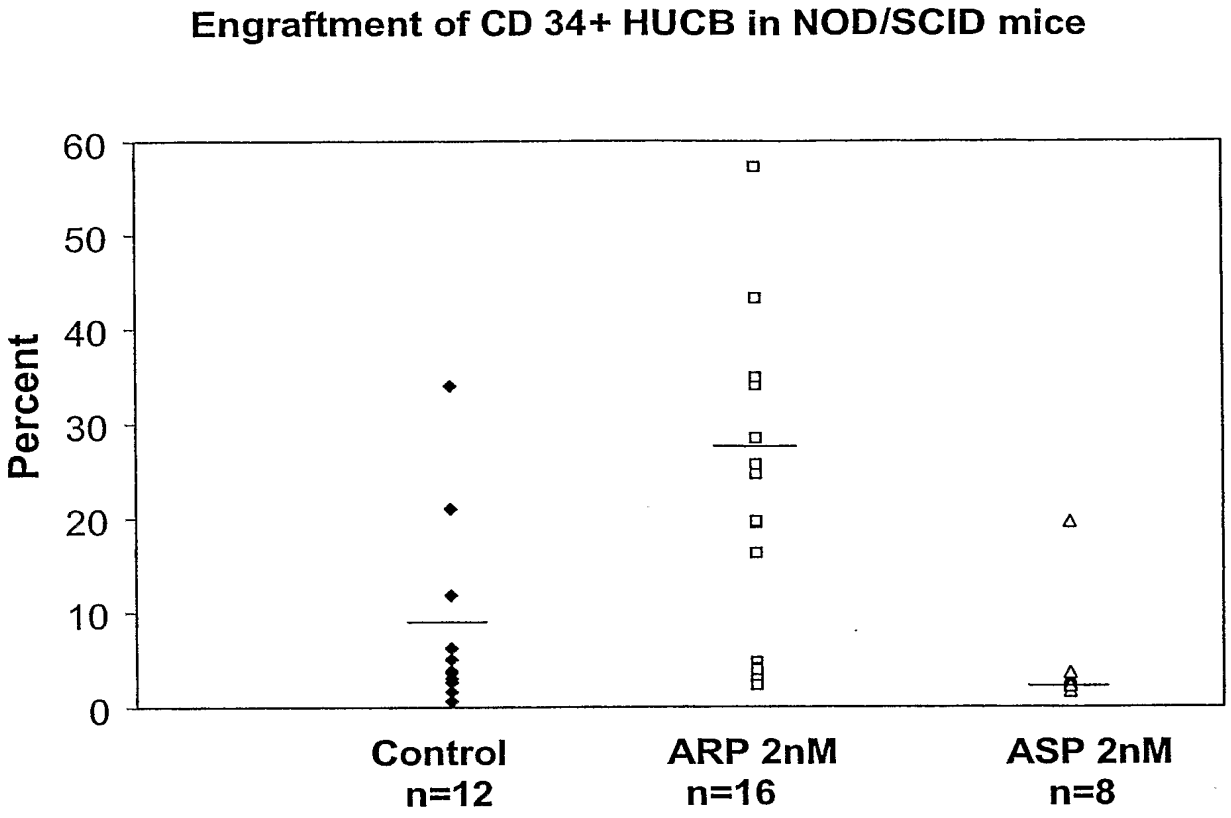
Engraftment of CD 34+ HUCB in NOD/SCID mice
Percent
60
50
40
30
20
10
0
Control
n=12
ARP 2nM
n=16
ASP 2nM
n=8

Fig. 14